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The Spectral Response Curve of Rods Determined by the Method of Electrostimulation

By

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Introduction

The retinas of most vertebrates contain two different types of visual receptors, cones and rods, which are, in the duplicity theory, thought to mediate the photopic and the scotopic vision respectively. According to Hecht,[1] this double structure of the human retina is responsible for the Purkinje phenomenon that the relative luminosity of the spectrum varies with the adaptation state of the retina. Based upon the result obtained by his microelectrode technique, Granit[2] interpreted the Purkinje shift in the same line as did Hecht. The recent work of Wright[3] also supports the idea of the two different carriers of the Purkinje shift.

On the other hand, Dartnall[4] has recently advanced a hypothesis that the apex of the luminosity curve is displaced towards longer wavelength by one or more of the breakdown products of visual purple which modify the amount of light absorbed.

Although the former idea by Hecht, Granit and Wright has generally been accepted, we have very little information about the mechanism in which the function of rods is replaced by that of cones in light adaptation. For elucidation of this mechanism it seemed important to investigate the behavior of rods in the state of light adaptation. Fortunately, the method of electrostimulation has provided a means to isolate the rod-response from the cone-response.

In the present investigation we attempted, therefore, to study how the spectral distribution of rod-responses changes with alterations in intensities of spectral lights.

Experimental

Method

The method consisted in measuring the electrical sensitivity of the

eye after exposure to a brief illumination.

The electrical stimulus used was a single constant current pulse of 100 msec. in duration. The stimulating electrodes were a pair of silver electrodes placed slightly above the eyebrow and on the homolateral temple.

After a preliminary dark adaptation of about 20 minutes, the eye was exposed to spectral light for 40 msec. and exactly 4.5 seconds later the eye was stimulated with an electrical pulse to determine its electrical sensitivity, E. The stimulating voltage was reduced step by step from a level high enough to cause a distinct electrical phosphene to the level at which the subject could no more distinguish an electrical phosphene from the background of intrinsic light of the retina.

In order to determine one threshold value it was necessary to repeat such a trial from 20 to 40 times. Successive trials were made at an interval of about 10 seconds. For control, the electrical sensitivity, $E_\circ$ at the resting level, was determined without a preceding flash of light. The effect of the light stimulus was expressed in terms of ζ which is defined by the formula: $\zeta = 100(E-E_\circ)/E_\circ$.

The value of ζ determined in this manner provides us with a measure of rod-responses for the following reasons. As reported previously by Motokawa *et al.*[5-6] and by Oikawa,[7] the ζ -time curve or excitability curve for rods attains its maximum at 4.5 seconds from the beginning of the light stimulus. As the cone-processes proceed more rapidly than the rod-process, the two kinds of processes can be studied separately.

An opaque glass disc of 0.5 cm. diameter, placed at a distance of 30 cm. from the eye served as a target, the visual angle being 1°. The spectrum used in this experiment was an equal-energy spectrum. The intensity level of the spectrum was given in terms of physiological units. One unit was an intensity 0.56 times as high as the threshold intensity measured at the dark-adapted fovea with red light (650 mμ) of 1° in visual angle. The intensity of the spectrum used could be raised as high as 145 units. The energy was made equal by adjusting the width of the collimator slit of the spectroscope to the prescribed values computed from the known spectral energy distribution of the light source. For further details the papers by Motokawa[8-9] should be consulted.

Results

Spectral response curves of rods obtained from different parts of the retina at various intensities are summarized in Fig. 1. In this figure, ζ-values determined in the manner described above, are plotted as ordinates against wavelengths as abscissas.

All curves show their most prominent maxima at about 510 mμ.

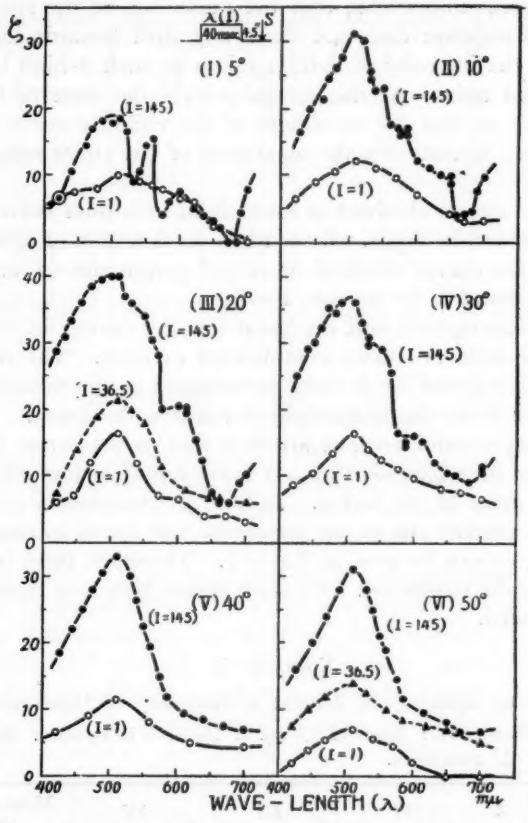

Fig. 1. Spectral distribution curves of rod-response obtained from various parts of retina at three different levels of light intensity. One physiological unit: An intensity 0.56 times as high as the threshold intensity measured at the dark-adapted fovea with red light (650 mμ) of 1° in visual angle. Ordinates: Percentage increase in electrical excitability of the eye over resting level determined 4.5 sec. after a flash of 40 msec. Abscissas: Wavelengths of pre-illuminating spectral lights. Spectrum used was an equal-energy spectrum.

The stronger the intensity of light the higher the maxima of the curves. It must be emphasized that the maxima of our curves lie at the same part of the spectrum as that of the scotopic visibility curve. In other words, the most effective wavelength to elicit a light sensation is also most effective to elicit the response determined by our method. From this fact it is obvious that the quantity ζ concerns the rod-mechanism.

It is of great importance that the maximum of the spectral distribution of rod-response does not show any shift towards longer wavelengths when the intensity of light is raised to such a high level as 145 in physiological units. At this intensity level the state of the eye becomes photopic so that the maximum of the visibility curve is found at about 560 mμ. Nevertheless the maximum of our curve remains at 510 mμ.

The set of curves obtained at lower light intensities below 36.5 units is generally smooth in shape. Even when the intensity of light is as high as 145 units, the curves obtained at retinal peripheries 40° and 50° from the fovea are found to be smooth, too.

At the midperiphery and the parafovea the curves for the same intensity of 145 units are more complicated in form. The irregularities of the curves are found much more pronounced as the measuring retinal location moves from the midperiphery towards the fovea.

These irregularities are not artefacts due to the errors of measurements, because such irregularities are never found at low intensities and at peripheral areas of the retina. In fact, the standard deviation of 10 measurements carried out at the parafovea was found as small as 0.129 in terms of ζ, as can be seen in Table I. Therefore these irregularities are physiologically significant. We shall discuss later how these irregularities are produced.

TABLE I

Experiment Showing the Degree of Accuracy of Measurements

Pre-illumination: Light of 575 mμ of 145 units in intensity. Retinal location: 5° from fovea.

Session	I	II	III	IV	Mean and standard deviation
ζ-values	4	4.5 3.5 4	4.5 4 4.5	3.5 4 3.5	4.0 ± 0.129

Much evidence has been accumulated that ζ-values determined at 4.5 seconds from the onset of illumination represent a reliable measure of rod-responses.[5-6)10)], A close resemblance between the spectral distribution of our quantity ζ and the scotopic visibility curve is one of these evidences. For the purpose of more scrupulous survey of the similarity, one of our curves, that is, the broken curve in Fig. I (III) and the scotopic visibility curve determined by Hecht & Williams[11)] are represented in Fig. 2 in such a manner that the value of each maximum is arbitrarily put at 100. As can be seen in this figure, the wavelength of each maximum coincides with each other, but our curve does not descend to the zero-level at the longer and shorter wavelength ends of the spectrum as does

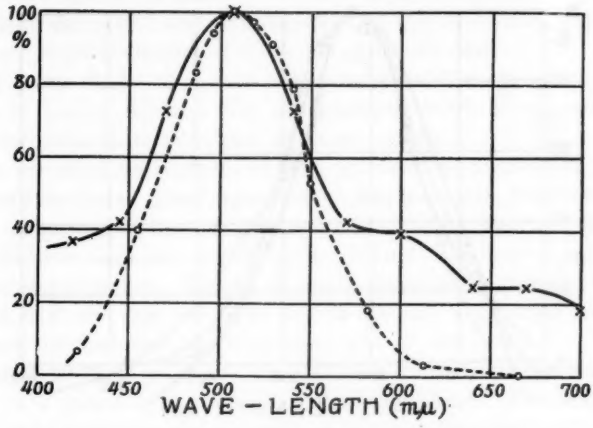

Fig. 2. Spectral distribution curve of rod-response obtained from the retinal location of 20° from fovea at an intensity of 36.5 units (full line) and scotopic visibility curve by Hecht and Williams (broken line). Both curves are drawn in such a way that values of 510 mμ coincide with each other.

the visibility curve. However it seems that our quantity ζ will become proportional to the visibility by a simple correction, that is, by subtraction of a certain definite value—say 8—from our ζ-value.

In reality, the spectral distribution of our corrected quantities of ζ was found in satisfactory agreement with the scotopic visibility curve as shown in Fig. 3. In this figure the continuous curves connecting crosses and solid circles refer to the data obtained at 30° and at an intensity of 1 unit and to those obtained at 50° and at an intensity of 36.5 units respectively. For comparison, the scotopic visibility curve by Hecht & Williams and that by Walters & Wright[12] are also represented in the broken lines connecting empty circles and triangles respectively in the same figure. This fact indicates that our quantity ζ is in linear relationship to the scotopic visibility.

Thus, the two kinds of curves coincide with each other at least at lower intensities, but at higher intensity levels some difference can appear between them as are shown in Fig. 4. In this figure similar curves for an intensity of 145 units obtained at 40° and 50° are compared with the scotopic visibility curve by Hecht and Williams.

At the part of longer wavelengths a close agreement is found, but at the shorter wavelength end of the spectrum our curves are much higher than the visibility curve. This discrepancy is greater for the curve obtained at 40° than for that obtained at 50°. Such disagreement at higher intensities may be an expression of influence of light adaptation, though

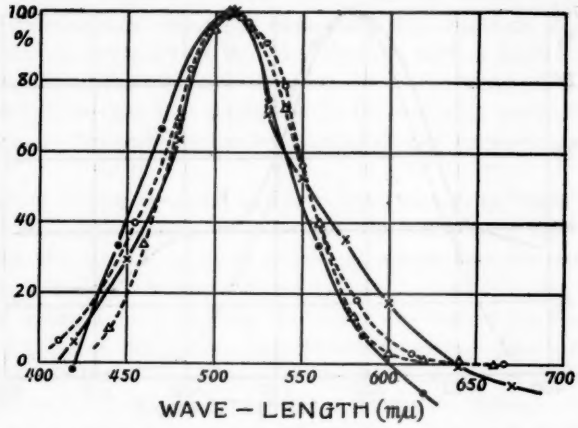

Fig. 3. Spectral response curves of rods (corrected) at lower intensities of light in comparison with scotopic visibility curves. Full lines connecting crosses and solid circles respectively originate from the curve for I=1 at 30° and from that for I=36.5 at 50° in Fig. 1. Scotopic visibility curve determined by Hecht and Williams and that by Walters and Wright are represented by a broken curve connecting empty circles and by that connecting empty triangles respectively.

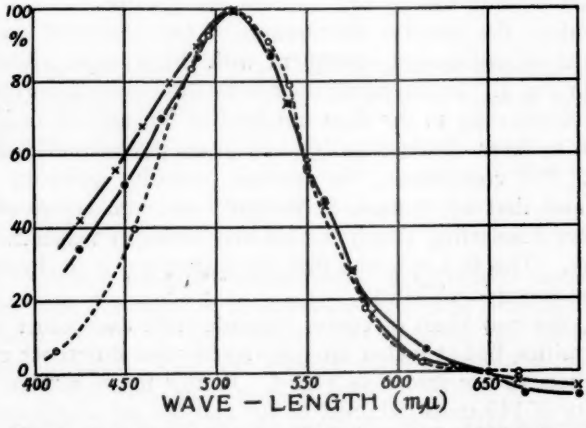

Fig. 4. Spectral response curves of rods (corrected) at higher intensities in comparison with scotopic visibility curve by Hecht and Williams. Full curves connecting crosses and solid circles respectively originate from the curve for I=145 at 40° and from the curve for I=145 at 50° in Fig.1.

its mechanism is not known to us at present.

As mentioned above, some notches appear in the spectral distribution

curves of rod-response at higher intensities of spectral lights when parafoveal parts of the retina are illuminated. As can be seen in Fig. 1, such notches are generally found at 670, 575 and 530 mμ.

There is no doubt that this phenomenon is closely connected with excitation of cones, because the phenomenon takes place only under experimental conditions favorable to cone-activity.

There are some grounds to ascribe the notches mentioned above to an inhibitory action of cones upon rods. For example, the notch found at 575 mμ is produced because the spectral light of wavelength 575 mμ is more effective than any other lights around this wavelength for inhibiting the rod-response. In fact, it can be demonstrated in the following experiment that the spectral light of 575 mμ has a stronger inhibitiory action upon the rod-response than the light of 560 mμ.

In Fig. 5 the rod-response at a retinal part 5° from the fovea is represented as a function of intensities of illumination. The response increases to some extent as the intensity of illumination rises, but decreases above a certain intensity. The critical intensity at which the decrease

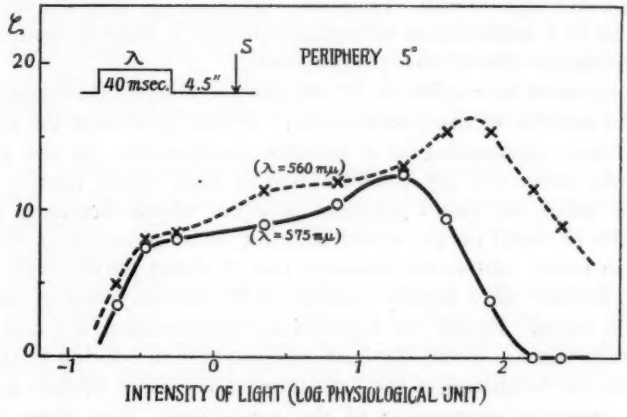

Fig. 5. The dependence of magnitudes of rod-response to lights of 560 mμ and 575 mμ upon logarithm of light intensity. Experimental procedure is shown in inset. The curve for 575 mμ begins to decrease at a lower intensity than that for 560 mμ.

begins is lower for the spectral light of 575 mμ corresponding to the notch under consideration than for the other light of 560 mμ. This means that the light of 575 mμ has a stronger inhibitory action upon the rod-responses.

In our experiments, such notches are generally found at red, yellow

and green parts of the spectrum. This fact suggests that there are at least three kinds of color receptors, red, yellow and green in the human retina. We expected that a notch could be found at the blue part, too, because there should be a blue receptor in our retina. In reality, however, no notch has been found at the blue part of the spectrum.

If the blue receptor is so much akin to the rod as Roaf[13] and recently Willmer[14-19] emphasizes, we shall be able to understand the reason why the blue part of the spectrum has no such inhibitory action upon the rod-response as do red, yellow and green parts of the spectrum.

Discussion

As mentioned above, Dartnall tried to account for the Purkinje shift quantitatively from relative amounts of visual purple and indicator yellow, and it seems that his attempt was successful so far as the shift of the luminosity maximum is concerned.

If his interpretation were correct, it might be expected that light adaptation should bring about a shift of the maximum of our rod-response curve. As a matter of fact, however, no such shift whatever could be seen in our experiment. Therefore, the Purkinje shift cannot be accounted for as a modification of rod-activity and it must be assumed that cones participate also in this phenomenon.

The question now arises as to why the rod-activity decreases in light adaptation relative to the cone-activity. Wright presented the following photochemical explanation as a possible mechanism: As the intensity of the light increased, the concentration of both visual purple in rods and some unknown visual pigment in cones would decrease, the decomposition of visual purple would continue to be greater than the visual pigment in cones, and so the recovery rate of visual purple might be expected to decline more rapidly relative to the recovery rate of the visual pigment in cones. Hence, the equilibrium concentration of visual purple, and the rod-response would diminish progressively in relation to the cone-response as the brightness of the light increased. Thus, Wright suggested that the apparent suppression of the rod-activity takes place through the mechanism of light adaptation.

There are, however, some difficulties in explaining our data in such photochemical terms. As has been shown above, the spectral response curves of rods determined with spectral lights of high intensities have conspicuous notches at certain definite parts of the spectrum, and these notches are certainly beyond the experimental error. On the other hand, we know that the absorption curve of visual purple is generally smooth all over the spectrum, no such irregularities being found as are seen in our rod-response curves obtained at higher intensities. If our response

curves were to be interpreted from the photochemical point of view, it would be extremely difficult to reconcile one fact with the other.

The other alternative presented by Wright is that a depression of rod-activity in light adaptation would be caused by some inhibitory action of cones upon rods, and it is rather easier to understand our data from this point of view.

The notches of our response curve mentioned above are situated at the red, yellow and green parts of the spectrum, and this fact is also in line with the hypothesis of inhibition, because the inhibitory action of cones should be maximal at the parts of the spectrum at which each kind of cone-receptor is activated maximally.

Summary

The rod-response was isolated from the cone-responses by the method of electrostimulation, and the effect of intensities of light was investigated upon the spectral distribution curves of rod-response obtained at various parts of the retina.

1. The maxima of all curves were found at the same spectral region as that of the scotopic visibility curve, that is, at about 510 mμ.

2. Even when the intensity of light was raised to such a high level that the eye became surely photopic, the maxima of the curves did not show any shift. In other words, the rod response curves show no Purkinje shift.

3. The curves obtained at lower intensities were smooth and coincided well with the scotopic visibility curve by a simple correction, but some irregularities were found on the curves obtained from parafoveal areas at high intensities.

4. The magnitude of rod-response increased with increasing intensities of light, but was depressed above a certain intensity. This fact and the notches on the rod response curves obtained with spectral lights of high intensities were ascribed to an inhibitory action of cones upon rods.

Prof. K. Motokawa furnished guidance and helpful criticism, for which we express here hearty thanks.

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Innervation of Heart in Dog

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Many histological studies on the innervation of human and animal hearts have been published since Kölliker[1] reported on his study on frog's heart in 1862, leading to many notable observations. Among these studies, the one conducted by Prof. Seto[2),3)] of this Laboratory on human heart is worthy of special commendation, and his observations and deductions have been fully endorsed by such eminent neurologists as Stöhr[4),5)] and Hermann[6]. Ábrahám,[7] in 1940, made a study on the same subject, but the imperfect silver method he had in command debarred him from arriving at the extreme minuteness which Seto's researches could reveal.

Treading in the paths of these illustrious predecessors, I made a similar study with the canine hearts as my objective. I hoped I could contribute something in elucidating the difference of human and canine hearts by a collation of my observations with the results of previous studies. In fact, there were studies on canine hearts including those belonging to partial studies, by Smirnow,[8),9),10)], Dogiel[11),12)], Valedinsky[13)], Cajal[14)], Michailow[15),16),17)], Worobiew[18)] and Lawrentjew[19)], but there seems to be many a point of doubtful accuracy in their observations.

My materials were taken from adult canine hearts, with special attention to the atria, and fixed for a long time in 10% neutral formol, which I cut into 40μ frozen sections and stained with Seto's silver impregnation practiced in this laboratory. The large series of beautiful slides obtained thus I subjected to a close microscopic study.

Experimental

Individual Observations

The Gerlach's[20)] so-called basal plexus or the Dogiel's[21)] subepicardial plexus, found in the atria and provided with ganglia in places, corresponds to the primary plexus composed of most powerful nerve bundles according to Seto's study on human hearts, being formed at the boundary between the epicardium and the myocardium. This observation holds also by the

canine heart I studied. These primary plexus goes over into the secondary plexus consisting of thinner nerve bundles found in both the epicardium and myocardium, which again changes into the more fine meshed tertiary plexus consisting of still thinner bundles. In the canine heart, the above primary plexus is also formed around the Tawara's node and the His's bundle. Some fine nerve fibres are seen penetrating as far as into the endocardium.

It has been known that the external nerve fibres coming into the primary plexus originate in the vagus and the sympathetic nerves, the former consisting of thin non-medullated parasympathetic fibres and stout medullated sensory fibres. The sympathetic and parasympathetic fibres could be here hardly distinguished histologically.

The distribution of the ganglia in the human heart has been minutely described by Francillon,[21] and Seto[2] confirmed his description. In my canine heart, I found a distribution of ganglia almost identical as that in human heart. So, the majority of the ganglia were found in the dorsal wall of the atria and at the entrance of the venae cavae in the heart, being found, however, sporadically in the other parts of the atria. For example, some nerve cell groups are found in the sulcus interauricularis, septum atriolum, valvula Thebesii, and septum fibrosum atrioventriculare, in particular, in its periphery.

Seto[2] succeeded in observing existence of nerve cells also in the secondary plexus in the myocardium of human atria cordis. This observation is very exceptional, according to Hermann[6], but in my adult canine specimen, frequent aggregations of nerve cells were found in the myocardium, as ascertained by Seto, and Dogiel[12] and Valedingsky[13] too report of their discovery of nerve cells in the myocardium of various mammals.

The development of the plexus and ganglia in canine atria is little in arrear in comparison with that in human heart. The large ganglia have generally a connective tissue capsule, while no such capsule is found accompanying the small cell groups (Fig. 1). The ganglia are rarely formed in the nerve bundles, the majority being found around the bundles closely touching them. The external nerve fibres coming in are comparatively small in number. The size of the ganglia is not necessarily proportional to the nerve bundles connected therewith, and often enough, a large ganglion is in contact with a thin nerve bundle.

The microscopic construction of the nerve cells in the canine atria has been described by Smirnow[11] and Lawrentjew[11], but perhaps due to the imperfection of their staining methods, their illustrations show only a very simple formation of the cells. According to my study, the nerve cells in canine atria are classifiable into the Dogiel's Type I and Type II,

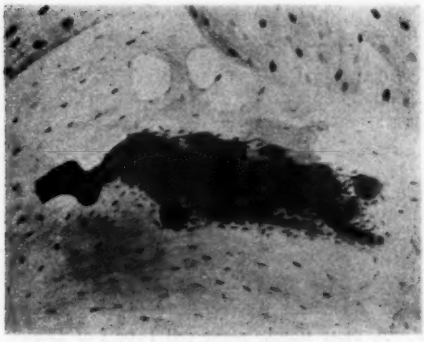

Fig. 1. A small ganglion seen in the pericardium of the left atrium cordis in dog. a small nerve bundle; no existence of connective tissue capsule is seen in the surroundings of the nerve cell group. Seto's silver impregnation, ×200.

as is the case with the nerve cells in human atria (Seto). However, whereas in human hearts the large majority of nerve cells are said to belong to the Type I, in canine hearts, cells of Type II being also present comparatively in a great number.

The principal difference between the nerve cells in canine and human atria consists in that the former are smaller in size and simpler in formation. In canine hearts, the nerve cells are provided with a very ill-developed connective tissue capsule, and even this poor integument is often denied them. The mantlecell-plasmodium is much smaller by canine than by human atria, and consequently the cell nuclei in it are also far fewer in number. The number of the nerve processes emerging from the nerve cells is also fewer than by man, as described hereunder, and the short processes are simpler in their ramification and termination.

The Dogiel's so-called nerve cells Type I are represented by cells with short processes terminating in the mantlecell-plasmodium. The long processes thereof are mostly single per cell, but more than two long processes per cell are not rare. The number per cell of the short processes is decidedly smaller than by human atria, and their ramification and terminal formation are much simpler. In Fig. 2, for example, a Type I cell sends out 2 or 3 short processes which terminate after only a very short and simple course in the plasmodium. The Type I cell seen in Fig. 3 has a few more short processes than in the foregoing, but their course and terminal formation are much simpler. In the two examples above, the short processes are seen emerging from any indefinite spot of the entire surface of the cell bodies. Thus, the plasmodium around the cells is nearly equal in thickness in all its parts.

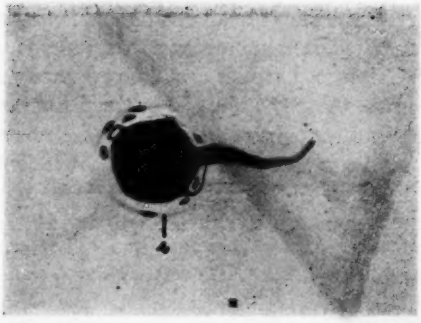

Fig. 2. A most simple nerve cell Type I of Dogiel provided with 2–3 short nerve processes found in the pericardium of the canine atrium. The number of cell nuclei in the mantlecell-plasmodium is very small; b connective tissue capsule. Same staining, ×500.

Fig. 3. A simple nerve cell Type I of Dogiel with several short processes ending in the mantlecell-plasmodium seen in the pericardium of the canine atrium. Cell nuclei in the plasmodium are comparatively numerous. Same staining, ×500.

But the case is different with the cell in Fig. 4. Herein, all the nerve processes emerge from one pole of the cell body, and the short processes end in the plasmodium that is formed here in a cone containing numerous plasmodium-nuclei aggregated in this part. Such Type I cells of specific conformation have been reported by Seto in human atria, but this peculiar sort of cells shows a much simpler formation in my canine heart than that in man.

Dogiel's cells Type II are represented by the cells with short processes running out through the connective tissue capsule to end in the vicinity of the mother cells. In my dog specimen, the nerve cells belonging to the Type II have in general one or two long processes and 3 to 6 short processes. The latter end sharply without undergoing a complex branching, after running out through the connective tissue capsule, as shown in Fig. 5.

Seto[2] has observed that the short processes emerging from a Type II

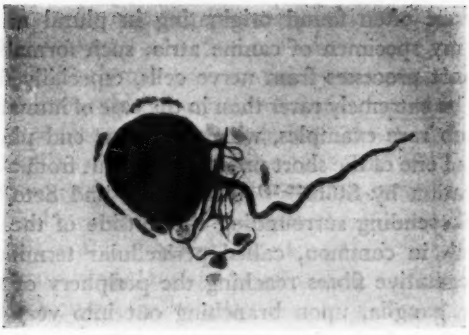

Fig. 4. A specific Type I cell of Dogiel seen in the pericardium of the canine satrium. All the short processes emerge from the one pole of the cell body, to end in the conically formed plasmodium rich in specific cell nuclei. Same staining, ×500.

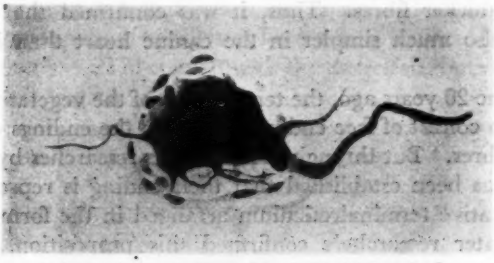

Fig. 5. A nerve cell Type II of Dogiel found in the canine atrium. All the short processes run out through the connective tissue capsule. Same staining, ×500.

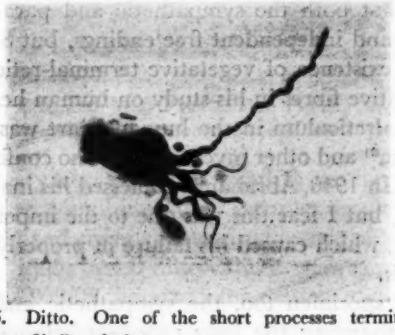

Fig. 6. Ditto. One of the short processes terminates in a conical neuro-fibril end-plate.

cell in the human atria often end in bulb-like or oval terminal plates, and some of them end buried in another nerve cell body. Moreover, such

end formations are often found originating in plural number from one single cell. In my specimen of canine atria, such formation of terminal plates on the short processes from nerve cells, especially from cells Type II, was found to be extremely rarer than in the case of human heart. Fig. 6 shows one of such rare examples, where only one end plate is formed at the termination of one of the short processes sent out from a cell Type II.

The observation by Stöhr[22-25], Reiser[26),72)] and Seto[2)] revealing the forming of netlike ending surrounding the outside of the plasmodium of of the nerve cells in common, called pericellular terminalreticulum, by the incoming vegetative fibres reaching the periphery of the cells in the vegetative nerve ganglia, upon branching out into very fine fibres, has been amply confirmed also by my study on the ganglia in the canine atria. However, in canine heart, the pericellular terminalreticulum is rarely formed by so colossal a number of minute fibres as by man, but in majority of cases, is formed by a more dispersed interlacing arrangement of somewhat thicker fibres. Thus, it was confirmed that this terminal formation is also much simpler in the canine heart than in the human heart.

Up to some 20 years ago, the termination of the vegetative nerve fibres was deemed to consist of free endings, much as the endings of sensory and motor nerve fibres. But through the laborious researches by Stöhr, Reiser and Seto, it has been established that their ending is represented by the so-called vegetative terminalreticulum arranged in the form of spread-out net. Many later researchers confirmed this proposition, and the fact has been now verified beyond any dispute. This holds true also of the termination of the vegetative fibres in the heart, both sympathetic and parasympathetic. Formerly, Smirnow[10)], Michailow[17)], Lawrentjew[18)] and Boeke[28)] reported that both the sympathetic and parasympathetic nerve fibres had separate and independent free endings, but Seto[2)] was the first in establishing the existence of vegetative terminal-reticulum common to both kinds of vegetative fibres in his study on human heart in 1936. This discovery of terminalreticulum in the human heart was highly lauded by Stöhr[24),25)], Hermann[6)] and other investigators, who confirmed the existence of such reticulum. In 1940, Ábrahám[7)] confessed his inability to affirm the observation of Seto, but I fear this was due to the imperfection of staining method he adopted, which caused his failure in properly staining the most delicate nerve fibrils.

In my canine specimen too, the sympathetic and parasympathetic nerve fibres, accompanying the long processes originating in the nerve cells in the atria, run out of the plexus peripherally, the bundles of these nerve fibres gradually decreasing in size, forming anastomosis with other nerve bundles at frequent intervals, and further decreasing in size. Then,

the nerve fibres go over into the Reiser's preterminal fibres, finally to end in the terminalreticulum.

The terminalreticulum is represented by the nerve cord in which the minutest neuro-fibrils which cannot be divided further, spread out reticularly. The terminalreticulum contains Schwann's nuclei in places, and stands in tactile control over almost any kind of tissue cells.

The terminalreticulum is found in a large number in all the layers of the atria. In the epicardium, it is spread out in the connective tissue and fat tissue, and in the myocardium, along the heart muscle fibres. In the latter part, it runs also along the blood vessels, especially, the capillaries, standing in a common control relation thereto, and never ends freely. In a similar manner, the terminalreticulum in the heart always acts in common supply over the controled cells.

The terminalreticulum is formed most abundantly and in a most typical formation in the walls of the arteries. The terminalreticulum running among the heart muscle fibres is also comparatively numerous, showing the common supply of capillaries here, too (Fig. 7). In this case, the terminalreticulum comes in contact with the tissue cells but does not penetrate into the cell bodies.

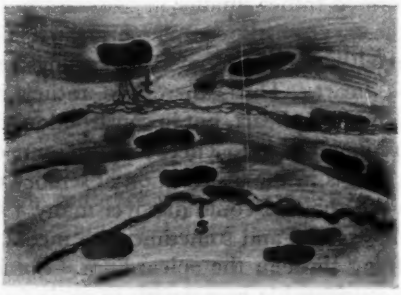

Fig. 7. Vegetative terminalreticulum (t) in the myocardium of the canine atrium. s sensory nerve fibre running with vegetative preterminal fibres of Reiser. Same staining, ×800.

As for the sensory nerve endings found in the hearts of animals, Smirnow[8] was the first in describing rather complex branched terminations in the amphibian heart and complex arborized terminations in the epicardium of rats and cats in 1895, which observations were confirmed by Dogiel[11], Michailow[15],[16], in 1907/08 described the existence of capsulated and uncapsulated branched terminations in the endocardium and epicardium in horse. However, as the above observations were made in specimens stained with methylene blue, there seems to be some justification for further scrutiny to determine their accuracy. Lawrentjew[19] (1929) observed

complex arborized endings in his study on the hearts of dogs and cats with silver method. Lawrentjew and Gurwitch-Lasowskaya[29] (1930) reported their discovery of a certain type of sensory nerve endings in the connective tissue in the atrioventricular bundle in rat. As may be readily surmised from the above enumeration, the study on the sensory nerve terminations in the hearts of animals may be termed as yet very sporadic.

On the other hand, in the case of human hearts, there is the thoroughly dependable research by Seto[2], which brought many detailed observations on the terminal formations of the sensory fibres to light. I also succeeded in observing numerous sensory terminations of various types in my very beautifully stained preparations of canine heart and in collation with the findings of studies on human heart (Seto), have arrived at the following results.

Somewhat against my expectations, I found many thick medullated nerve fibres in the heart of dog. These I take to be sensory fibres derived from the vagus in their majority, as Seto describes such fibres in the human heart. These fibres all terminate freely, and their terminations can be divided into the unbranched, the simple branched terminations with 2 or 3 rami and the terminations of Type I connected with the falling reflex of blood pressure formed rather smaller in size than in human heart. But I could not find those extremely complex branched endings described by Smirnow[8] and Dogiel[11], nor the glomerular endings reported by Michailow[15],[16] in horse's heart. Thus, there are rather numerous sesnsory terminations found in canine heart, but in construction, these are far simpler than those found in human heart. There are also some differences between canine and human hearts in the distribution of sensory terminations.

In addition to the above, I could discover many a sensory termination penetrating into the externa and sometimes the media of the branches of aa. coronariae running through the epicardium. Some of these terminations even showed considerable complexity in their formation.

Now, the unbranched terminations are the simplest of the sensory endings. A stout medullated fibre, after losing its myelin, runs the winding course peculiar to sensory fibres, showing some change in size, generally to end sharply. Such terminations are found in any of the layers of the atria, with special predominance in the myocardium.

Fig. 8 shows a typical unbranched termination found in the myocardium of a dog. In some cases, these terminations show a characteristic ansiform course, that is, a very simple glomerular formation, as shown in Fig. 9. Unbranched terminations are found often in the epicardium as well, attention being specially invited to the existence of some of them running in close contact with the basis of the endothelial cell layer of the epicardium. Often some of the unbranched terminations found in the

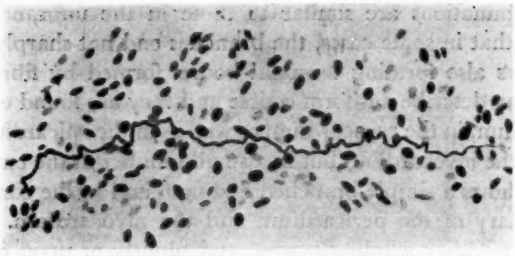

Fig. 8. A typical unbranched sensory termination found in the myocardium of canine atrium cordis. Same staining, ×200.

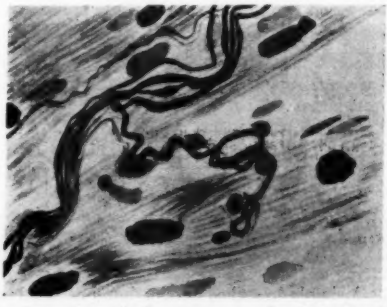

Fig. 9. An unbranched sensory termination showing a simple ansiform course found in the myocardium of the canine atrium. Same staining, ×800.

endocardium are observed as formed by stout sensory fibres meandering in close contact with endothelial cells, as shown in Fig. 10.

The nature and running course of nerve branches in the simple

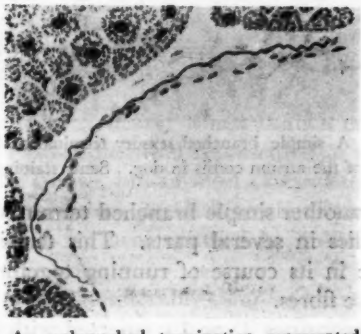

Fig. 10 An unbranched termination represented by a thick sensory nerve fibre and ending direct under the endothelial cell layer of the endocardium. Canine atrium cordis. Same staining, ×500.

branched terminations are similar to those in the unbranched terminations, except that in some cases, the branches end not sharply but bluntly, and sometimes also forming terminal bodies formed by fibril dissolution. These also may describe ansiform course and they are found everywhere, in the endocardium, in the myocardium as well as in the epicardium, especially abundant in the myocardium but in a small number in the endocardium.

Fig. 11 shows a simple branched ending found in the connective tissue at the boundary of the pericardium and the myocardium. The 2 or 3 rami, after a peculiar winding course, end bluntly or upon completing an ansiform course. Fig. 12 shows a simple branched termination found in the myocardium, with the rami showing a rather remarkable change in

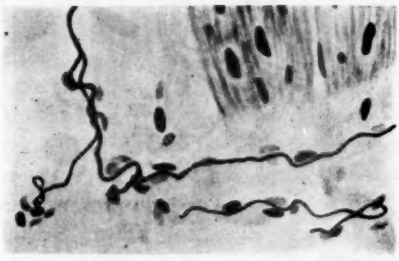

Fig. 11. A simple branched ending found at the boundary between the pericardium and myocardium of the atrium cordis dextrum in dog. Terminal branches end sharply or bluntly. Same staining, ×500.

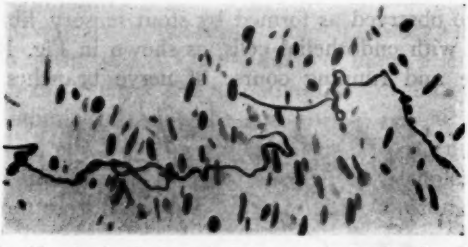

Fig. 12. A simple branched sensory termination found in the myocardium of the atrium cordis in dog. Same staining, ×320.

size. Fig. 13 shows another simple branched termination, showing formation of terminal bodies in several parts. This termination is formed by a thick sensory fibre in its course of running together with a few other sensory and vegetative fibres.

The sensory terminations Type I connected with the depressor reflex, discovered by Sunder-Plassmann[30] and de Gastro[31] in the carotic sinus, have been found by Seto[2] in human arcus aortae as well as in the human

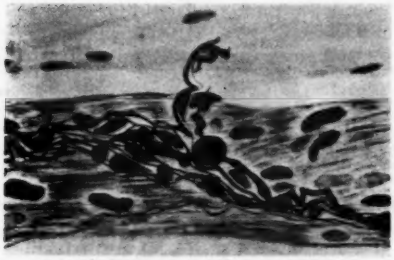

Fig. 13. A simple branched sensory termination formed in the running course with the other nerve fibres, having special neurofibril bodies. Myocardium of the left atrium in dog. Same staining, ×500.

atria cordis[3], in particular, in the connective tissue of the myocardium. I could also observe such terminations, quite similar to the human counterparts in formation, in the canine endocardium, especially in the endocardium adjacent to the limbus fossae ovalis.

These endings are formed, as shown in Figs. 14 and 15, by sensory

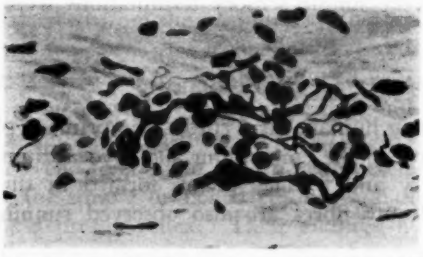

Fig. 14. Termination Type I connected with the falling reflex of blood pressure seen in the endocardium in the surroundings of fossa ovalis of the canine atrium cordis. Many special end-plates are seen along the termnial branches and in their tips. Same staining, ×800.

fibres with very stout trunk fibres, which branch out in numerous rami changing in size after branching and represented as strong fibres. Sometimes in their running course, but in most cases at the tip of the terminal branches there are formed very peculiar terminal plates due to fibril dissolution. These endings are accompanied by numerous globular special nuclei.

In human heart, some considerably complex branched sensory terminations have been found around the small veins in the myocardium (Seto)[2] but in my canine heart, sensory terminations were extremely rare around the veins. However, sensory fibres were found running along the small

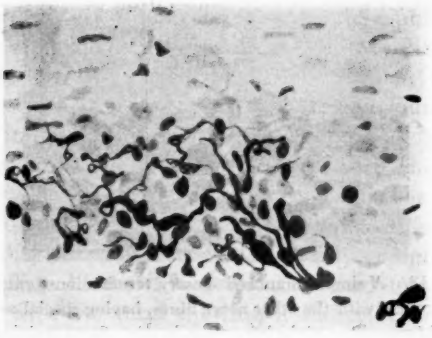

Fig. 15. Ditto.

arteries everywhere, which end mostly in comparatively simple branched terminations.

Seto has often found in the human heart extremely complex sensory terminations ending around the heart muscle fibres, but in my canine specimen, I found no such complex terminations replated to the muscle fibres, although very simple sensory terminations were not rarely found around them.

Summary

In canine, as in human, atria cordis, the primary plexus is formed at the boundary between the epicardium and myocardium, which goes over into the secondary and tertiary plexus formed in the epicardium and myocardium. Minute fibres are also observed running into the endocardium.

The incoming nerve fibres running into the primary plexus originate in the vagus and sympathetic nerves, the former consiting of non-medullated fibres, so that the distinction between sympathetic and parasympathetic fibres is not possible histologically.

The distribution of nerve cell groups found in the plexus in the epicardium is also similar by human and canine hearts. Ganglia are mainly found in the dorsal walls of the atria and the entrance of the venae cavae into the heart, but sporadically in all other parts of the atria. In canine heart, as in human (Seto), nerve cell groups are found also in the secondary plexus in the myocardium.

Nerve cells in the canine atria can be also classified into the cells of Dogiel's Type I and Type II, of which the latter exist somewhat more abundantly by dog than by man. The nerve cells in canine atria are much smaller in size and simpler in construction than those in human heart, e.g., the pericellular connective tissue capsule being poorly developed, often

even to total disappearance, the mantlecell-plasmodium being limited in size and the plasmodium-nuclei very much poorer in number. The number of nerve processes is also smaller than in human heart, especially with the nerve cells Type I, and the ramification and terminal formation of the short processes are much simpler.

The plasmodium surrounding the nerve cells Type I is generally aligned in a uniform thickness, but as is the case in human heart, frequently the plasmodium is found especially developed at one pole of the cell. But the arrangement of short processes branching and ending therein is far simpler than in man. In nerve cells Type II, the number of short processes is rather smaller than in man, and the terminations thereof more rarely end in special plates.

The terminations of incoming nerve fibres approaching the intragangliar nerve cells are represented by pericellular terminalreticulum, as in man, but these are much simpler than in man in construction. In dog, rather thick fibres are arranged in a comparatively dispersed interlacing mesh.

The termination of vegetative nerve fibres is represented by the terminalreticulum in canine atria as in human atria. This comprises the three kinds of elements derived from the incoming sympathetic and parasympathetic fibres and the long processes from the nerve cells, consists of nerve cords formed by extremely minute fibrils arranged reticularly, ends never freely, and stands in tactile supply over any of the controled cells.

A considerable number of medullated sensory fibres is seen running in canine atria. These end freely, and in form, can be classified into the unbranched endings, the simple branched endings and the endings Type I connected with the depressor reflex. These endings, however, are always much simpler in formation that in man. Their localization is also somewhat different from that in man. Beside the above kinds of terminations, there are found branched sensory endings in the walls of the branches of aa. coronariae cordis.

The unbranched terminations are found in the epicardium, the myocardium and the endocardium of the atria, but in special abundance in the myocardium. They end mainly sharply, but sometimes also upon completing an ansiform course. It is noteworthy that such endings are often formed close beneath the endothelial cell layers of the epi- and endocardium.

In the simple branched endings consisting of 2 or 3 rami, the branches end generally sharply, but some end bluntly or either in a terminal body formed by fibril dissolution or describing a simple ansiform course. These endings are found in all the layers of the atria, but tare particularly abundant in the myocardium and few in the endocardium.

The sensory terminations Type I connected with the depressor reflex are also found in canine atria, particularly in the endocardium around the fossa ovalis. They are poorer in scale by dog than by man (Seto), but the nature of the trunk fibres and the rami and the formation of special terminal plates through fibril dissolution are nothing different from those in man.

Unlike the case in human heart, sensory endings are rarely formed around the small veins, and if any, their terminations are very simple. But the number of sensory fibres running along the small arteries is very large, all ending in simple branched terminations. No complex sensory terminations are found surrounding muscle fibres in the canine atria.

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Histological Study of Olfactory Bulb in Man

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The histological study of the olfactory organ has been made by so many investigators in the field, since so many years ago, that the subject seems to have reached a final stage and the theories concerning the transmission of stimuli in the olfactory system well established, so as to preclude all questioning thereagainst.

However, lately Saito[1] of this laboratory dared to doubt the accuracy of the histological picture of the olfactory mucosa accepted hitherto and taking his materials from human adults and 10th month embryos, subjected the sections of the parts, stained with the excellent Seto's silver impregnation, to a minute microscopic scrutiny, resulting in bringing to light a histological picture quite contradictory to the accepted one—an epoch-making achievement. According to the observations of Saito,[1] the human olfactory epithelium is not a special one containing the so-called bipolar olfactory nerve cells, as so many researchers since the days of Shultze[2] (Cajal,[3] van Gehuchten and Martin,[4] Brunn,[5] Köliker,[6] Lenhossék,[7] Retzius[8] and Kolmer[9] among others), but only a variety of stratified columnar epithelium widely found in epithelia in other parts. The spherical cells found in this epithelium are not nerve cells, but are epithelial cells, and no such large-sized nerve cells such as said to be observed by Kolmer,[9] have been found in the epithelium. Consequently, it was presumed that the olfactory nerve fibres must have their nerve cells located in the brain, in particular in the olfactory bulb.

I undertook to make a histological research of the human olfactory bulb, to succeed Saito[1] in his study on the olfactory mucosa, and in comparison with the results of the past studies, to obtain an authentic histological picture of this part. My materials of study consisted of olfactory bulbs from adult human brains, which I fixed for a long time in 10% neutral formol, cut into 40 μ sagittal and transverse frozen sections and stained with Seto's silver impregnation. The series of numerous beautiful preparations I subjected to a minute microscopic study and arrived at many

obervations utterly divergent from the histological picture depicted by my predecessors, which I will detail hereunder.

Experimental

Individual Observations

Many studies of the olactory bulbs, chiefly with animal bulbs as materials, were conducted and reported by many investigators since many scores of years ago, beginning with Leydig,[10] Owsiannikow,[11] Walter[12] and Clarke[13] and succeeded by Meynert,[14] Ganser,[15] von Gudden,[16] Golgi,[17] Henle,[18] Schwalbe,[19] Obersteiner,[20] Zuckerkandle,[21] van Gehuchten and Martin,[4] Kölliker,[6] Retzius,[8] Edinger,[22] Cajal,[3] Smith,[23] Read,[24] McGatter[25] and Kuntz,[26] of which that by Cajal[3] seems to have been looked upon as representative. But even his study could not claim much in improvement upon those by Owsiannikow[11] and Clarke,[13] except that his histological pictures of the glial cells and olfactory glomeruli were somewhat more distinct.

According to Cajal,[3] the glomeruli in the olfactory bulb are composed of olfactory nerve fibres and the dendrites of the so-called mitral cells, and the stimuli from the olfactory mucosa are received by the latter to be transmitted to the olfactory center through the axons of these cells. This theory has been accepted by van Gehuchten and Martin,[4] Retzius,[8] Kölliker[6] and other later investigators. Cajal[3] also recognized existence, beside the mitral cells, of nervous granule cells, of nerve cells with a short axon in the olfactory bulb and of pyramidal cells in the olfactory tract. He thought the mitral cells were neurons of second order and the pyramidal cells neurons of third order. There are also some efferent fibres running into the olfactory bulb, which end in granule cells, which again are connected with the dendrites of the mitral cells through their axons. The olfactory bulb is divided into five layers—the layer of peripheral nerve fibres, the glomerular layer, the molecular layer, the mitral cell layer and the granular and deep-seated nerve fibre layer, counting upwards from the bottom.

Kuntz,[26] in his Neuro-Anatomy, 1936, published somewhat different opinions from Cajal[3] on the subject of human olfactory bulb. He says, the central part of the olfactory bulb consists of neuroglia, surrounded by the medullated fibres running into the olfactory tract. The exterior of the olfactory bulb is formed of gray matter covered by unmedullated fibres coming from the olfactory nerve. The gray matter is composed of large and small mitral cells and granule cells, the large processes of the former reaching the exterior surface to interlace with the terminal branches of the olfactory nerve fibres and form glomeruli there. The

axons from both the mitral cells enter the deeper layers to go over into olfactory tract fibres, which run toward the center sending out collaterals in the course. The granule cells on the exterior surface give out dendrites reaching glomeruli in their vicinity and axons to other glomeruli, uniting the glomeruli into groups. The granule cells in the deeper layers send out their axons to the surface, which commingle with the dendrites and collaterals of the mitral cells. The efferent nerve fibres coming into the olfactory bulb through the anterior commissure were supposed to end in contact with the granule cells in the deeper layers.

In summarizing the most important of the past theories connected with the transmission of olfactory stimuli, we may say that the glomeruli are formed by the commingling of the terminal branches of the axons from the olfactory cells in the olfactory mucosa with those of the dendrites from the mitral cells. The mitral cells receive here the stimuli transmitted from the olfactory cells and forward them to the highest olfactory center in the brain through their axons. Beside the above, axons from the granule cells also play a part in the formation of glomeruli.

However, upon a minute scrutiny of my beautifully stained preparations, I have arrived at observations widely different from the past theories and opinions upon the formation of the glomeruli as well as the nature of mitral and granule cells. Besides, I wish to emphasise the fact that there were found in the olfactory bulb many vegetative nerve cells which have never been described by any past researcher. According to my observations, let me first point out that the olfactory bulb consists of four layers, namely, the layer of olfactory nerve fibres, the glomerular layer, the gray matter layer and the layer of granule cells and nerve fibres, from the bottom upwards, before entering upon the nervous composition of the olfactory bulb and the olfactory tract.

1. Layer of lofactory nerve fibres. This layer is the part in which the olfactory nerve originates and is formed of numerous small bundles of nerve fibres arranged irregularly, as shown in Figs. 1 and 2. Saito[1] has already subjected the microscopic construction of the peripheral olfactory nerve fibres to a thoroughgoing observation. I also found that the olfactory fibres in this layer are utterly different from other sensory cerebrospinal nerve fibres in their appearance.

Whereas the common cerebrospinal sensory fibres are represented by individual medullated thick fibres which never come into nervous anastomosis in their courses, the small bundles of olfactory fibres are never represented by groups of independently running nerve fibres, as seen in the past descriptions, but by somewhat oblonged mesh-formation of undescribably peculiar, extremely minute, neuro-fibrils showing more or less change in size. That is, the olfactory bundles are composed of

minute reticules, whose nerve elements are by my impregnation somewhat weakly stainable to take a characteristic red color.

Mingled with the mesh forming fibres, there is found also a very small number of minute independently running dark-purple staining nerve fibres, but these also, according to Saito,[1] come into frequent anastomosis with the mesh fibres, so that there could be no functional difference between these two kinds of fibres. So, he states, the latter isolated fibres belong to the olfactory nerve, alike the mesh forming ones. In the bundles of olfactory fibres there are found many spindle-shaped, club-shaped and oval glial cells.

2. Glomerular layer (Figs. 1 and 2). This layer is represented by 1–3 layers of glomeruli, lining the upper side of the olfactory fibres layer. The proper elements constructing the glomeruli are the weakly red staining most minute neuro-fibrils formed in the reticules, which however, are arranged finer and more regularly than those in the olfactory fibres layer.

The connection of the glomeruli with the olfactory fibres is evidenced in Fig. 3, wherein it may be distinctly observed that small bundles of the olfactory fibres penetrate into the glomeruli, branch out, and their neuro-fibril nets dissolve into the reticules of glomeruli. However, it is my impression that the vice versa is nearer the truth, that is, the glomerular neuro-fibril nets go over into the bundle of olfactory fibres, gradually gaining size in their course. In the glomeruli, the number of glial cells is very small, some glomeruli containing no glial cell at all. As described hereunder, very stout dendrites are seen running into the glomeruli from the mitral cells.

3. Gray matter layer (Figs. 1, 2 and 4). This layer is visible with considerable distinctness under low magnification, as a light colored layer. In appearance it recalls the molecular layer in the cortex of the cerebrum or cerebellum and that is perhaps the reason why Cajal[3] named it the molecular layer. However, under high magnification, or better under oil immersion, this is seen as consisting of minute reticules of weakly red staining neuro-fibrils of minutest size, seemingly of vegetative nature, mingled with equally weak staining nerve cells scattered among them. Glial cells are also found therein in a considerable quantity. In consideration of such a histological picture, I feel it most appropriate to call this layer the gray matter layer.

The nerve cells found herein may be classified in two kinds, the one corresponding to the so-called mitral cells and the other belonging morphologically to the vegetative nerve cells. The morphological distinction of the two is very easy, as given below. The so-called mitral cells, under the staining methods used by Golgi,[17] Cajal,[3] Kölliker,[6] Retzius,[8]

← Fig. 1. Horizontal section of human olfactory bulb. f layer of olfactory nerve fibres; g glomerular layer; n gray matter layer; k granule cells layer; o layer of nerve fibres. Seto's impregnation, X 50, reduced to 2/5.

Fig. 2. Sagittal section of human olfactory bulb. f layer of olfactory nerve fibres; g glomerular layer; n gray matter layer. Same staining, X 100, reduced to 1/2. →

← Fig. 3. Sagittal section of human olfactory bulb, showing the relationship between the glomeruli and the small olfactory nerve bundles. Cell nuclei are all glial cell nuclei. Same staining, X 700, reduced to 2/5.

Fig. 4. Sagittal section of human olfactory bulb, showing the gray matter layer and the glomerular layer. One of the dendrites from a bulbus cell is seen entering into a glomerulus. Small-sized nerve cells are vegetative cells. Small cell nuclei having no cell bodies are glial cell nuclei. Same staining, X 200, reduced to 1/3. →

Obersteiner,[20] Edinger,[22] Smith[23] and Kuntz,[26] were seen as cells of trapezoid shape, with their cell bodies staining uniformly black, sending our several thick dendrites toward the glomeruli and with a single thin axon running out of the rounded top of the cell bodies.

In going through my studies, I came to feel that, because of the incomparably complicated histological figure of the olfactory bulb, it must have been utterly impossible to clarify these intricacies with any of the hitherto available staining methods as weapon. In my ideal impregnated preparations so beautifully stained, wherein the minutest of the neurofibrils are sharply distinguished, the mitral cells showed figures so widely different from those described by the above mentioned researchers, that in general contour they do not remotely resemble mitres. I wish to propose the name of " bulbus cells " instead of the inappropriate " mitral cells." These bulbus cells are found at a distance from the glomeruli in the granule cells layer described below and in the periphery of the anterior end of the olfactory tract, as well as in the vicinity of the glomeruli.

These bulbus cells are shaped like elongated spindle or triangle or are stellate, as the sensory nerve cells in the spinal cord (Suzuki),[27] only rarely showing rounded appearance. Some of them are bipolar (Fig. 5), but the majority of them are multipolar (Figs. 6, 7 and 8), sending out 3–6 nerve processes. These processes do not emerge singly from one pole like the limbs of an octopus, as shown in the illustrations of the so-called mitral cells, but grow with confounding irregularity. The cell

← Fig. 5. A bipolar bulbus cell seen in the gray matter layer of human olfactory bulb. Same staining, X 700, reduced to 2/5.

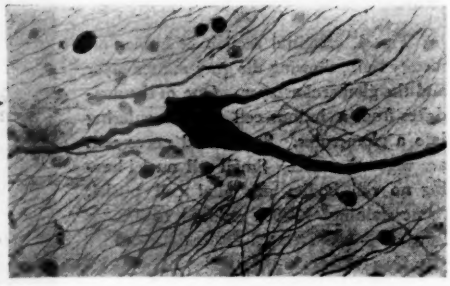
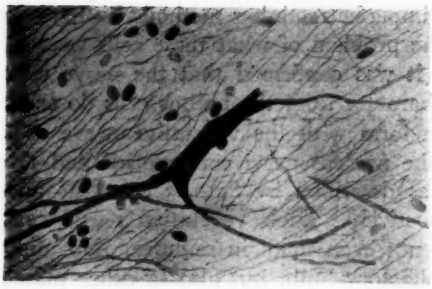


Fig. 6. A stellate multipolar (4 →) nerve processes) bulbus cell in the gray matter layer of human olfactory bulb. Same staining, X 700, reduced to 2/5.



← Fig. 7. A spindle-shaped multipolar bulbus cell in the same place. Same staining, X 700, reduced to 2/5.

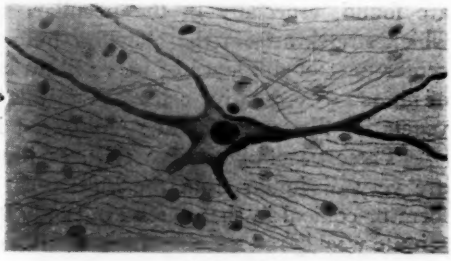


Fig. 8. A stellate multipolar bulbus cell in the same place. Same staining, X 700, reduced to 2/5. →

nucleus is situated at the center of the cell body, and spherical, elliptical or oval in shape and rather small in size.

In the cell bodies, weakly red staining minute neuro-fibrils are found forming reticules, which go over into the nerve processes. The processes, both the dendrites and the axons, are fibres of considerable size. The dendrites sometimes divide into 2–3 branches in their courses and diminish gradually in size as they run to the periphery, ending in the above described glomeruli to help building them (Fig. 4). However, the discovery of the part played by the dendrites in the formation of the glomeruli is accompanied by extreme difficulties, because, the tips of the dendrites running into the glomeruli are sometimes extremely fine and the course of the dendrites is often considerably long, so that it is very frequently impossible to determine whether the extremities of dendrites are in sight or not.

On the other hand, the axons of these cells have been described as rather thin fibres sending out collaterals and ending in so-called pyramidal cells (Cajal) in the past reports. However, according to my observations, these axons are not much different in size from the dendrites, and after a considerably long course, they turn into deeply dark-violet staining medium sized conspicuous nerve fibres and run toward the center.

Vegetative nerve cells. A few researchers in the past have reported the existence of small nerve cells beside the mitral cells in the so-called

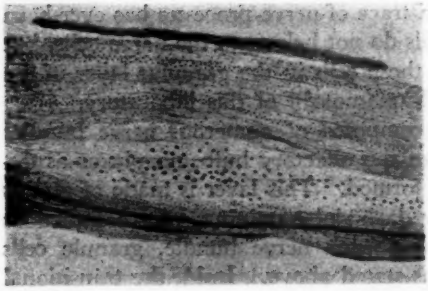
mitral cell layer, but due to the imperfect staining methods hitherto in use, nothing was established on the problem of what these cells were in their nature. In my study also, it was confirmed that the nerve cells different in their morphological character from the bulbus cells do exist. These cells are entirely similar in form with the vegetative nerve cells in the nucleus nervi hypoglossi, the nucleus nervi coulomotirii and the gray matter in the spinal cord, as described by Sato,[28] Atsuki[29] and Suzuki[27] of this laboratory respectively. Such vegetative nerve cells existing in the olfactory bulb are not even small in number. Not only in the gray matter layer of the olfactory bulb, but also in other places are found, especially in the peculiar, spindle-shaped nucleus (Fig. 9), which stretches from the olfactory bulb to the olfactory tract along the center part of the layer of nerve fibres and is formed by an aggregation of numerous vegetative cells. From this vegetative nucleus, some minute vegetative fibres are found running out in bundle from its both ends.

These vegetative nerve cells, as illustrated in Figs. 10, 11 and 12, are very dissimilar from the bulb cells. They are of rounded spindle-form or conical in shape, send out only 2–4 nerve processes, contain very minute weakly red staining neuro-fibrils and have a very large cell nucleus, in comparison with their cell bodies approximately at the center. Thus, the vegetative nerve cells found in the central nerve system are represented by very delicate looking cells with a oversized cell nucleus. The vegetative nerve cells in the olfactory bulb are either bipolar or tripolar in majority, quadripolar or quinpolar cells being found only occasionally.

4. Layer of granule cells and nerve fibres. This layer, according to Cajal,[8] is formed of plexus of medullated nerve fibres and numerous groups of tiny nerve cells found in the cavities in the plexus. These tiny cells or the so-called granule cells were said to receive the stimuli from the efferent fibres running through the olfactory tract peripherally and to transmit them to the mitral cells through their xaons.

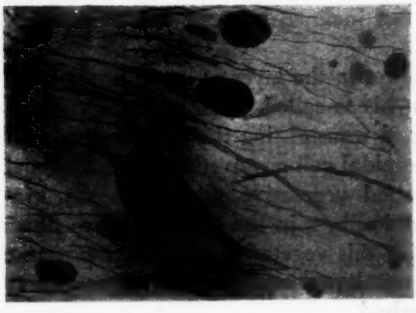
In my ideal sections, I found many small round cell nuclei grouped parallel to the long axis of the olfactory bulb in long bands touching the above described gray matter layer or arranged alternatively with the bundles of nerve fibres stretching in a similar way. However, the cell groups lying on the inner side of this layer are weaker in development than those situated more to the exterior side, while, in general, the nerve bundles are more strongly developed as we go deeper into the interior. I agree in naming this layer the layer of granule cells and nerve fibres.

These granule cells were looked upon as small nerve cells having many nerve processes since the days of Cajal, but in my silver preparations, no such histological picture was observable. The cell nucleus of chiefly rounded shape is located in very little cell bodies, no neuro-fibrils are



← Fig. 9. Spindle-shaped vegetative nucleus existing in the layer of nerve fibres in the transitional part of the olfactory bulb into the olfactory tract in man. Horizontal section. Same staining, X 50, reduced to 2/5.

Fig. 10. Vegetative nerve cells seen in the gray matter layer of the human olfactory bulb. Same staining, X 1500, reduced to 2/5. →



← Fig. 11. Vegetative nerve cells seen in the gray matter layer of the human olfactory bulb. Same staining, X 1500, reduced to 2/5.

Fig. 12. Vegetative nerve cells seen in the vegetative nucleus in the hinder part of the human olfactory bulb. Same staining, X 700, reduced to ½. →

proved in the cell bodies and not a trace of nerve processes has ever been found. So it is clear that these cells do not belong to the nerve cells, but are glial cells in their nature, like those cells in the granular layer of the cerebellum, vividly described by Yamamoto[8)] of this laboratory.

The layer of nerve fibres is composed of numerous nerve bundles running parallel to the long axis of the olfactory bulb, no plexus as described by Cajal and others being formed. This layer is made of groups of independently running, strongly dark-violet staining, very conspicuous medium sized nerve fibres. Around each nerve bundle, granule cells are distinctly arranged in bands, as stated above. Inside the transitional part between the olfactory bulb and the olfactory tract there lies the above mentioned spindle shaped nucleus of vegetative nature formed parallel to the long axis at the inner part of the layer of nerve fibres, and the minute vegetative fibres are seen entering and leaving both its ends.

The strongly stainable nerve fibres constructing the layer of nerve fibres are doubtlessly represented by the direct continuation of the axons of the bulbus cells running afferently, but it is noteworthy that the transition is sometimes very indistinct.

I could not find anything upon which to base my denial of the existence of efferent nerve fibres in the layer of nerve fibres as assumed by many researchers, because I could find nothing to mention touching the subject, in my preparations, but I feel inclined to deny the existence of such nerve fibres.

According to Cajal, the nerve fibres in the layer of nerve fibres originate in the mitral cells and end in his so-called pyramidal cells which constitute the neurons of third order in transmitting their received stimuli to the center. However, as the pyramidal cell group mentioned by Cajal must correspond to the spindle-shaped vegetative nucleus in my preparations, and as the strongly stainable fibres in the layer of nerve fibres do not show any fibrous relation with this vegetative nucleus, I presume that the above statements of Cajal are based on misobservations due to the imperfection of his staining method.

As described above, I succeeded in obtaining some results following my minute scrutiny on the microscopic structure of the olfactory bulb, by means of my supremely ideal silver preparations, of which results I will summarize the most outstanding in the following.

The bulbus cells have one axon running afferently and some dendrites running peripherally. The latter first take part in the formation of the glomruli, and successively pass over into reticular bundles of olfactory nerve, which finally reach the epithelium of the olfactory mucosa. That is to say, the bulbus cells, like the sensory nerve cells constituting the cerebrospinal ganglia, are interpreted as forming the neurons of first

order connected with the olfactory sensation. Sensory cells other than these bulbus cells connected with the latter are hardly existent in the olfactory bulb, but vegetative nerve cells are found in large number in the olfactory bulb. Especially, it is noteworthy that there is a spindle-shaped nucleus of vegetative nature lying in the layer of nerve fibres between the olfactory bulb and the olfactory tract.

Summary

The olfactory bulb in man is divisible into the layer of olactory fibres, the glomerular layer, the gray matter layer and the layer of granule cells and nerve fibres, counting upward from the inferior side.

The layer of olfactory fibres is composed of many small bundles of olfactory fibres running irregularly. The olfactory fibres are not independently running fibres as are the other sensory fibres, but are represented by a peculiar mesh formation of weakly stainable, minute neuro-fibrils of various sizes. However, a small number of strongly stainable, independently running, minute fibres is also found among the above. There are also numerous glial cells in the olfactory bundles.

The glomeruli in the glomerular layer are represented by the regular mesh formation of neuro-fibrils finer than those in the olfactory bundles and go over into the small bundles of olfactory nerve, only rarely containing glial cells.

The gray matter layer is composed of minute weakly stainable neuro-fibril nets perhaps of vegetative nature, containing a large number of glial cells as well as scattered weakly stainable nerve cells. The nerve cells in this layer are classifiable into those corresponding to the so-called mitral cells and vegetative nerve cell. However, as the former hardly appear as rounded, mitre or octopus-shaped cells, I wish to propose the name of bulbus cells for them. These are shaped like elongated spindle or triangle, or are stellate, are multipolar with 3–6 nerve processes, having a rather small cell nucleus respectively, and their cell bodies contain weakly stainable neuro-fibril nets. Their processes, both the dendrites and the axons, are made of stout fibres, the former, sometimes after sending out 2–3 rami, lose in size as they run peripherally and finally run into the above mentioned glomeruli, where they play a part in the formation of the latter. This last observation has cost me some overwhelming difficulties. The afferent axons finally become strongly stainable conspicuous fibres and merge into the layer of nerve fibres.

Vegetative nerve cells are found not only in this layer but also in the vegetative nucleus described below in large number. These are rounded, spindle-shaped or conical cells, as those found in the brain and the spinal cord, contain weakly stainable delicate neuro-fibril nets and send out

2–4 nerve processes. It must be also mentioned that they contain a large cell nucleus in the midst of their cell body.

The layer of granule cells and nerve fibres is composed of numerous bundles of nerve fibres derived from the axons of the bulbus cells running parallel to the long axis of the olfactory bulb, and many round cells arranged alternatively with these fibre bundles. The granule cells are not multipolar nerve cells as accepted hitherto, but are glial cells as those found in the granular layer of the cerebellum (Yamamoto).

Cajal's so-called pyramidal cells are not sensory in nature, but actually correspond to the vegetative cells in the spindle-shaped vegetative nucleus stretched at the transitional part between the olfactory bulb and tract. Minute vegetative fibres are seen entering and leaving both the ends of this nucleus.

In short, the dendrites from the bulbus cells go out to play a part in forming the glomeruli, the latter go over into the olfactory fibres, which finally reach the epithelium of the olfactory mucosa, while their axons transmit the received stimuli to the highest olfactory center in the brain. consequently, the bulbus cells are interpreted as the olfactory neurons of first order corresponding to the sensory nerve cells in the cerebrospinal ganglia. No other sensory cells related with the bulbus cells are found in the olfactory bulb.

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Innervation of the Hard Palate in Cat and Hedgehog

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The histological study of the innervation of the hard palate of mammals has been tried by Elin[1], Bethe[2], Botezat[3] and Retzius[4] in older days and more recently by Ábrahám[5], with some noteworthy results, but there are some points yet of justifiable doubts on the subject. As for human hard palate, the studies by Kadanoff[6] and Kaji[7] attacked the subject, which Sakai[8] treated with exhaustive minuteness recently. Funabacshi[9] succeeded him with his excellent study on human embryos so that the knowledge on the innervation of human hard palate seems to have assumed quite a new aspect.

We undertook, in succession of the above mentioned studies, to study the same subject with one month baby cats and adult hedgehogs as objective, utilizing the newest silver impregnation perfected by Prof. Seto of this Laboratory, and collating the results with those of the studies on human palate, arrived at the following observations.

Experimental

Individual Observations

Let us preface our description on the innervation with histological pictures of the mucous membrane of the hard palates of our subject mammals. Lund[10] has divided the mucous membrane of the human hard palate into the fibrous marginal zone, the fibrous median zone, the adipose zone and the glandular zone, from the histological point of view, and Sakai[8] acknowledged the adequacy of the division. No such division has been reported on the histology of mammalian hard palates, but our observations revealed a similar division as applicable in our specimens. The fibrous marginal zone and the fibrous median zone correspond to the parts of the mucous membrane covering the transitional part from the hard palate to the gum and the region of the median raphe respectively, where

the tunica submucosa is lacking and the mucosa is firmly bound to the periosteum by connective fibres. The adipose zone occupies the frontal part of the hard palate, where the submucosa is composed of adipose tissue, and the glandular zone is situated on the hinder part of the hard palate, where the submucosa is full of mucous glands.

The nerves supplying the feline hard palate originate in rather stout nerve bundles running along the periosteum. These bundles send out rami into the glandular tissue in the glandular zone of the dorsal half and into the adipose tissue in the adipose zone of the ventral half of the hard palate, which branch out and anastomose among themselves to form the submucous plexus of large meshes. From these plexuses, minute rami are sent out into the tunica propria, which also branch out and anastomose mutually to form the plexus tunicae propriae (Fig. 1). In both the above plexus, no

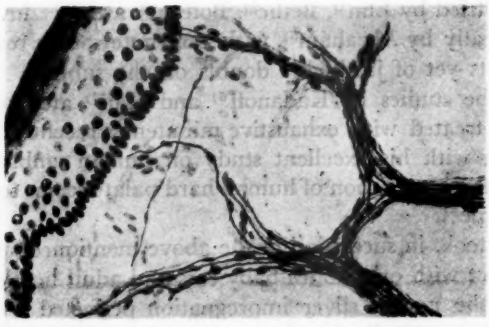

Fig. 1. Plexus laminæ propriae in the feline hard palate. Thick fibres are sensory fibres, thin fibres are vegetative fibres. Seto's impregnation, ×700, reduced to 2/5.

nerve cells are found existing, similar to the case with the human hard palate (Sakai[8] and Funabashi[9]). The plexuses are formed of thick medullated sensory nerve fibres and thin unmedullated vegetative fibres, the latter undergoing frequent anastomosis among themselves. Most of the sensory fibres terminate in the connective tissue papillae jutting into the epithelium, as in human palate. The development of the sensory fibres are always in proportion to the development of such papillae. According to the study by Sakai[8], the height of the epithelium in human hard palate increases as we go from the backward to the forward and very slowly from the medial to the lateral side, in disagreement from the results of Klein's study. The increase in height is especially marked in the transition from the marginal part to the gum. The development of papillae keeps abreast with the height of the epithelium, being poor in the glandular zone, be-

coming better in the adipose zone, and the marginal zone, existing in very tall though slender specimens. On both sides of the median line there are many strongly developed broad papillae, especially in the part ventral to the transverse folds of the palate. Among the low, poorly developed papillae in the glandular zone, some better developed oval papillae are found commingled.

The above observations on the epithelium and papillae of human palate are applicable to the cat in general. Only, the height of the epithelium and the development of the papillae are much lower in cat than in man, and no such large papillae, as observable in man, are ever found in cat. Such facts suggest that the sensory fibres in feline hard palate are in a much lower development than in human palate.

In fact, we found the sensory fibres in feline hard palate much inferior to those in human hard palate, both in quantity and formation. Into the smaller of the papillae, we found even no nerve fibre penetrating at all. Most of the sensory nerve fibres here are represented by sharply endling very simple unbranched terminations (Fig. 2). Into the larger papillae,

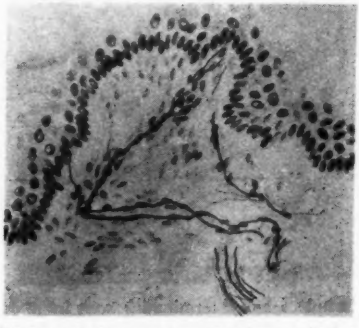

Fig. 2. Unbranched sensory terminations ending in a papilla in the feline hard palate. Thin fibres are vegetative fibres. Same staining, ×500, reudced to ½.

however, comparatively many sensory fibres are found penetrating, which sometimes arrange themselves confusedly in every direction and form indefinite terminations (Fig. 3), but otherwise also end in simple branched terminations. But we could not prove eixstence of such rather complex branched endings with terminal plates, as described by Ábrahám[5] in the hard palate in cow. The thin vegetative nerve fibres accompanying the thick sensory fibres end in the tunica propria in terminalreticulum (Fig. 4), which stands in tactile control over the capillaries and the basis of the epithelium in particular.

Intraepithelial fibres are also found in existence in the feline hard

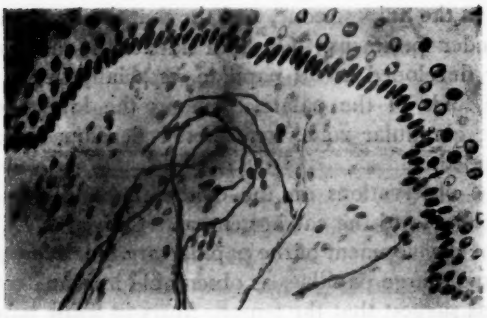

Fig. 3. Indefinite termination composed of many sensory fibres formed in a papilla. Feline hard palate. Same staining, ×700, reduced to 2/5.

Fig. 4. Simple sensory terminatins seen in a papilla. Feline hard palate. Some of sensory fibres pass over into intraepithelial fibres ending in the epithelium. t vegetative terminalreticulum. Same staining, ×100, reduced to ½.

palate. The development, however, of these fibres is also closely dependent on that of the papillae, the epithelium enclosing larger papillae containing better developed intraepithelial fibres. This situation has been proved by Sakai[8] and Funabashi[9] as obtaining in adult and embryonic human palates too. So, since the papilla formation in feline hard palate is much more backward than in human palate, the intraepithelial fibres in the feline palate are far poorer in development than in man, and no such complex branched endings formed of several branches as observed in the epithelium capping large papillae in human palate (Sakai[8]) have ever been found in our feline specimen, where the intraepithelial fibres are seen generally

running out in unbranched terminations, though in rare cases, the endings are branched in two rami. But many intraepithelial fibres running into the epithelium lining one large papilla are not rare (Fig. 5). We found no such complex branching and anastomosing intraepithelial fibres as described by Retzius[4] in the hard palate in cat. We presume that such were only artefacts manufactured by the imperfect staining he used.

The intraepithelial fibres are sensory fibres running into the papillae penetrating as far as the epithelium, and are never composed of such stout fibres as Ábrahám[5] described in the hard palate in cow. They are of course unmedullated fibres, of unvarying thinness and smooth surface, running through or between epithelial cells, reaching the surface of the epithelium, to end in or between epithelial cells in the vicinity of the cornified plate, sharply (Fig. 6) or with terminal nodes (Fig. 7). How-

Fig. 5

Fig. 6

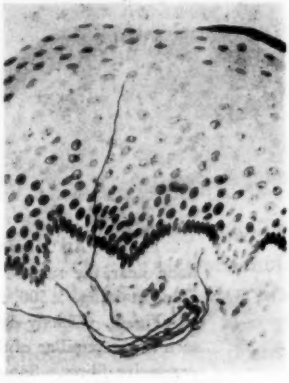

Fig. 5. Intraepithelial fibres ending in the basal layer of the epithelium. Feline hard palate. Same staining, ×700, reduced to 2/5.

Fig. 6. An unbranched intraepithelial fibre ending sharply in the vicinity of the surface layer of the epithelium. Feline hard palate. Same staining, ×500, reduced to ½.

ever, some of them end after an ansiform course in the basal layer of the epithelium, without reaching the upper layer, as shown in Fig. 5.

The plexus formation in the submucosa and the propria in the mucous membrane of the hard palate in hedgehog is almost similar to that in cat, but as the histological configuration of the hard palate in the former develops somewhat weaker than that in the latter, these plexuses are also in a somewhat lower stage of development. They are, however, made up of thick sensory fibres and thin vegetative fibres and do not contain any nerve cells in both of the cases alike.

The papillae are slightly poorer in development in hedgehog than in cat and major papillae are seen only in the plicae transversae, so that the sensory terminations formed in the papillae are found only in the plicae and mostly represented by simple unbranched terminations (Fig. 8), as found in cat. In major papillae, more than one sensory terminations are found coexisting, but the minor papillae are often provided with not a single nerve termination.

Fig. 7

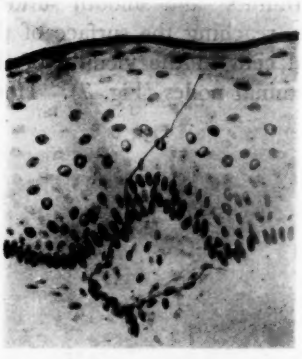

Fig. 8

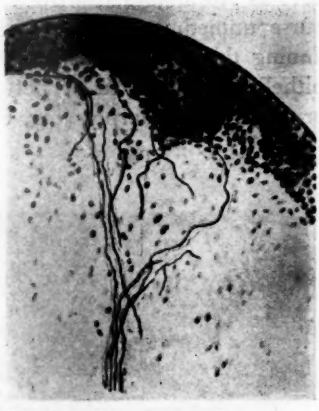

Fig. 7. An unbranched intraepithelial fibre ending mit a small node near the surface layer of the epithelium. Feline hard palate. Same staining, ×500, reduced to ⅓.

Fig. 8. Chiefly sharply ending unbranched sensory terminations seen in the papillae of the hard palate in hedgehog. Thin fibres are vegetative fibres. Same staining, ×400, reduced to 2/5.

The intraepithelial fibres (Fig. 9) are also similar to those in feline hard palate, the epithelium over large papillae being in most cases provided with intraepithelial fibres, while in general no intraepithelial fibre is found running through a small papilla into the epithelium. These intraepithelial fibres are uniformly thin and smooth-surfaced, ending sharply without branching, as in the feline hard palate, but only rarely reach the surface layer of the epithelium, most of them ending in the basal or the middle layer thereof.

Summary

The fine structure of the hard palates of the cat and the hedgehog is also similar to that in man, but in development the former is far in arrear to the latter, especially in the thickness of the epithelium and the size of

Fig. 9. Unbranched sensory terminations ending in a papilla and the epithelium of the hard palate in hedgehog. Same staining, ×600, reduced to ½.

the papillae in the mucous membrane. Consequently, the development of the sensory terminations formed in the papillae and of the intraepithelial fibres ending in the epithelium stands also in a much lower stage in these mammals than in man. The histological picture of the hard palate of the hedgehog showing a somewhat lower development than that of the cat, the development of the sensory nerve terminations is also to some extent poorer in hedgehog.

The sensory terminations in the hard palate in cat are chiefly formed in the major papillae, and mostly represented by very simple unbranched terminations. The intraepithelial fibres are also formed by sensory fibres penetrating into the epithelium from the large papillae, and also end mostly in simple unbranched terminations. These fibres are composed of unmedullated smooth-surfaced, uniformly thin fibres, run through or between epithelial cells and end sharply or with small nodes near the surface layer of the epithelium.

In the hard palate in hedgehog, the sensory terminations in the papillae as well as the intraepithelial fibres are all similar in formation and nature to those in cat, but are developed somewhat poorer than the latter both in number and in scale. In particular, the intraepithelial fibres here mostly end in the basal or middle layer, without reaching the surface layer, of the epithelium.

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Studies on the Mechanism of Alloxan Initial Hyperglycemia

I. A Role of Pituitary and Adrenal Gland in Connection with the Effect of Alloxan on the Blood Sugar

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The first report about alloxan diabetes was made by Dunn, Sheehan and McLetchie[1], then by Bailey and Bailey[2], and by Goldner and Gomori[3] in 1943. Given by intravenous injection into animals, alloxan induced a typical triphasic course of the blood sugar curve. Following a large initial hyperglycemia (1st phase) starting immediately after the injection which lasted a few hours, there came a fall in the blood sugar level (2nd phase). About 24 hours after the time of injection, the permanent hyperglycemia, the third phase showed up. This characteristic blood sugar curve was already noted short time after the discovery of alloxan diabetes in 1943, and confirmed by many investigators since then[4].

Various hypotheses have been presented to explain the mechanism of this initial hyperglycemia in relation to the endocrine organs and to the pathologic-anatomic changes in the islets of Langerhans. Namely, Dunn *et al*[1]. reported that the initial hyperglycemia may theoretically be due to excessive mobilization through the adrenosympathetic or to diminished combustion due to the lack of insulin. Goldner and Gomori[5] confirmed further evidence in support of this hypothesis about the initial hyperglycemia that failed to appear in adrenalectomized rabbits and in rabbits whose adrenal medulla has been destroyed by injection of formalin. Kirshbaum, Wells and Molander[6] showed the lack of the initial hyperglycemia in hypophysectomized or adrenalectomized rats, while the blood sugar curve in demedullated rats did not differ from the normal. Houssay, Orias and Sara[7] reported that this initial hyperglycemia was due to a direct effect of alloxan on the liver. Their experimental results showed that this hyperglycemia could not be found in hepatectomized dog, and became greater if alloxan was injected directly into vena portae instead of vena saphena. Duff[8], Iversen[9] and Okamoto[10] argued that it may

be due to the initial lack of insulin secretion from the islets of Langerhans destroyed by the injection of alloxan, but Banerjee[11], Goldner[12], Bailey[13] and Wrenshall[14] opposed this, because they observed the initial hyperglycemia even in pancreatectomized dogs or in rabbits with temporary occlusion of arteries to the pancreas.

As stated above, the various hypotheses have been suggested, but the fine mechanism of alloxan initial hyperglycemia is still remained unknown. I have been engaged in a series of studies to determine the mechanism of this hyperglycemia, which include the study on the influence of alloxan on the blood sugar in hypophysectomized, adrenalectomized and demedullated rats and the study on the adrenal ascorbic acid concentration of the rats after the injection of alloxan in order to reveal the role of pituitary adrenocortical system to the alloxan initial hyperglycemia.

Experimental

Materials and Methods

The adult rats weighing from 150 to 250 g. were fed with the fixed diet for two weeks. The alloxan monohydrate solution was prepared in concentration of 5%, and it was injected intraperitoneally into the rats in doses of 180 mg. per kg. of body weight.

Hypophysectomy was performed by the technique which Nakao described.[15] Experiments were done on hypophysectomized rats within few days after the operation. Before the alloxan injection the rats were fasted about 2 hours. After death or sacrifice, they were examined whether pituitary gland existed or not.

Same procedure was taken in a group of adrenalectomized rats.

In the demedullated group, one adrenal was demedullated and the other adrenal was totally removed. The demedullated rats were sdudied 10 to 45 days after the time of operation in order to wait the regeneration of the adrenal cortex. The rats were sacrificed on the 3rd day after the injection of alloxan and the adrenals were studied histologically.

The blood sugar was determined by Hagedorn-Jensen's method every 30 minutes or 1 hour during 10 hours after the injection of alloxan.

The male rats were used in order to measure the ascorbic acid concentration of adrenals. The ascorbic acid concentration of adrenals was determined by the Indophenol method in normal rats and in the rats killed 2 hours after the injection of alloxan solution and of physiological saline solution in the same amounts with alloxan solution.

Results

The results of the hypophysectomized rats are showed in Table I and

TABLE I

The Blood Sugar Changes after the Injection of Alloxan in Normal and Hypophysectomized Rats

		No. of rats	Alloxan	Blood sugar value (mg/dl) after the lapse in hrs.												
				0	½	1	2	3	4	5	6	7	8	9	10	24	48
Normal		10	+	99	117	139	182	209	181	127	104	79	57	48	44	120	289
Hypophysectomized	complete	5	−	91		88	85		82		82		80		78		
		5	+	89	82	70	62	54	49	45	31	42	36			75	169
	incomplete	6	+	98	135	187	235	245	205	160	129	106	80	68	54	109	369
Adrenalectomized		6	−	84			84		81		78		76		75		
		9	+	87	78	66	57	45	36	30							

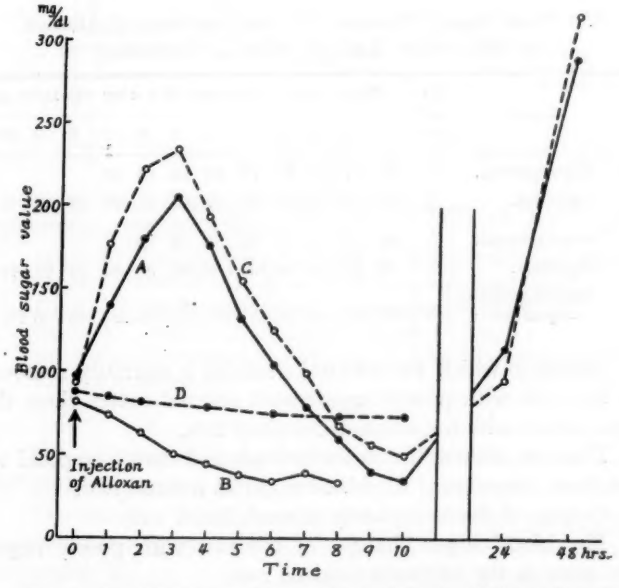

Fig. 1. The blood sugar changes after the injection of alloxan in normal (Curve A), in hypothysectomized (Curve B) and in incomplete hypophysectomized rats (Curve C). Curve D shows the fasting blood sugar in hypophysectomized rats.

Fig. 1.

Incompletely hypophysectomized rats (about 1/6–1/7 of the anterior pituitary lobe remained intact) respond with the typical triphasic reaction of the blood sugar as normal rats do. However, in hypophysectomized

rats, the initial hyperglycemia disappears and the blood sugar falls without fail immediately after the alloxan injection, and the degree of the fall is more marked than the hypophysectomized non-alloxan-injected rats. The hypoglycemic phase is very severe and often fatal in spite of repeated administration of large amounts of glucose. At 48 hours after the time of injection, the two survived cases show the hyperglycemia which is lower than the normal rats.

In adrenalectomized rats, the blood sugar responses to alloxan are same as in hypophysectomized rats.

The blood sugar changes after the injection of alloxan in the 24 demedullated cases are classified in four types in connection with the histological finding of the adrenals (Table II and Fig. 2).

TABLE II

The Blood Sugar Changes after the Injection of Alloxan in Rats under Various Adrenal Conditions

Medulla	Cortex	No. of rats	Blood sugar value (mg./dl.) after the lapse in hrs.													
			0	½	1	2	3	4	5	6	7	8	9	10	24	48
Partially demedullated	non-regenerat.	2	102	97	93	87	75	49	39	38	34					
	regenerat.	6	104	114	125	154	166	149	106	67	59	58	47	41	125	310
Completely demedullated	non-regenerat.	5	104	96	85	71	62	54	38	30						
	regenerat.	6	96	88	83	103	169	176	140	92	80	65	63	45	101	344
	hypertrophically regenerat.	5	105	92	86	92	145	193	245	280	320	348	378	477	740	1088

(A) Group in which the adrenal medulla is partially removed.

(1) The rats with poorly regenerated adrenal cortex show the same blood sugar curves with the adrenalectomized rats.

(2) The rats with well regenerated adrenal cortex respond with the typical triphasic reaction of the blood sugar as normal rats.

(B) Group of the completely demedullated rats.

(1) The blood sugar changes of the rats with poorly regenerated cortex are same as the adrenalectomized rats.

(2) In cases with regenerated cortex the blood sugar shows a fall immediately after the alloxan injection continuing from 30 minutes to 2 hours, and then the triphasic reaction appears.

(3) In cases with hypertrophically regenerated cortex the blood sugar comes down also during first 30 minutes and continues to be low for 2 hours, but the following initial transitory hyperglycemia is superimposed by permanent hyperglycemia without secondary hypoglycemic phase.

The ascorbic acid concentration of the adrenals of 3 groups are as

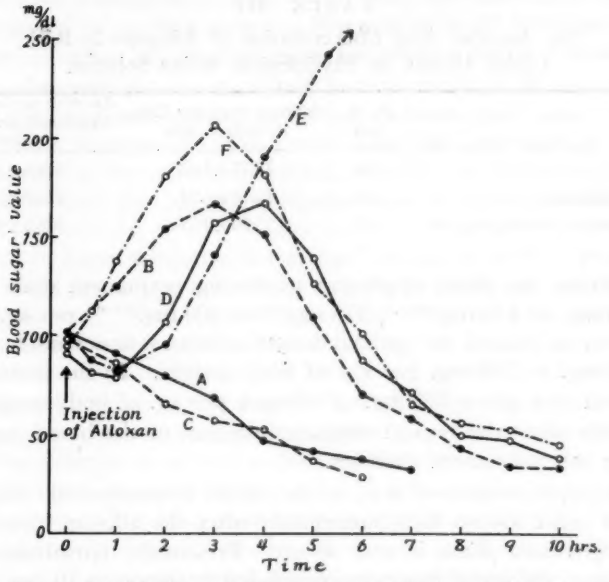

Fig. 2. The blood sugar changes after the injection of alloxan in rats under various adrenal conditions.

Curve A: partially demedullated rats with non-regenerated cortex
Curve B: partially demedullated rats with regenerated cortex
Curve C: demedullated rats with non-regenerated cortex
Curve D: demedullated rats with regenerated cortex
Curve E: demedullated rats with hypertrophically regenerated cortex
Curve F: normal rats

follows:—

(1) The ascorbic acid concentration of adrenals in the normal rats was 393.8±32.14 mg. per 100 g. of adrenals (group A).

(2) The ascorbic acid concentration of adrenals in the rats killed 2 hours after the alloxan injection was 229.0±19.68 mg. per 100 g. of adrenals (group B).

(3) The ascorbic acid concentration of adrenals in the rats killed 2 hours after the injection of physiological saline solution in the same amounts of alloxan solution was 388.0±41.29 mg. per 100g. of adrenals (group C) (Table III).

Discussion

The dose of alloxan used by the previous investigators to produce diabetes in rats are various in amounts. Especially in case of intraperito-

TABLE III

The Ascorbic Acid Concentration of Adrenals in Rats Given Alloxan or Physiological Saline Solution

	N. of rats	Adrenal (mg.) per 100g. of body weight	Ascorbic acid concentration (mg.) per 100 g. of adrenal
Normal	10	15.77±1.06	343.8±32.14
Alloxan injected	10	15.14±1.24	229.0±19.68
Physiol. saline solution injected	10	15.50±0.53	388.0±41.99

neal injection, the doses of alloxan producing permanent diabetes were from 100 mg. to 150 mg.[16),17)], 175 mg.[18)] or 200 mg.[19)-22)] per kg. of body weight, but in general the optimal dose of alloxan is now considered to be from 150 mg. to 200 mg. per kg. of body weight. In the present study the normal rats given 180 mg. of alloxan per kg. of body weight intraperitoneally show the typical triphasic reaction of the blood sugar followed by the permanent diabetes.

In hypophysectomized rats, as the initial hyperglycemia disappears, the blood sugar always falls immediately after the alloxan injection and this hypoglycemic phase is very severe. Previously, Kirshbaum *et al.*[6)] reported that, the initial hyperglycemia failed to appear in 10 rats in which alloxan in the doses of 100 mg. per kg. of body weight was given intravenously 2 hours after hypophysectomy. But, their report is lacking in details to discuss the rapid blood sugar changes after the alloxan injection, because the fasting blood sugar before the alloxan injection was showing a wide range of fluctuation and the blood sugar after the injection was determined at intervals of from 2 to 4 hours. Iversen[9)] reported that the alloxan initial hyperglycemia was repressed strongly in hypophysectomized rats, but many of his cases showed a slight hyperglycemia during from one to four hours after the alloxan injection, and only in 2 cases of 9 hypophysectomized rats the blood sugar fell immedately after the injection as shown in my cases.

It is considered very interesting and noteworthy that incompletely hypophysectomized rats (1/6–1/7 of the anterior pituitary lobe remained) respond with the typical triphasic reaction of the blood sugar as normal rats do. The difference of the blood sugar after alloxan injection between normal or incompletely hypophysectomized rats and hypophysectomized rats is very pronounced. These results indicate that the pituitary displays an important role in the alloxan initial hyperglycemia.

On the role of adrenal, Goldner,[5)] Iversen,[9)] Nagahama[23)] and Ishii[24)] showed that the initial hyperglycemia failed to appear in adrenalectomized rabbits. Kirshbaum *et al.*[6)] observed the same results in adrenalectomized

rats. So their conclusion is that the adrenal has something to do with the alloxan initial hyperglycemia. Houssay *et al.*,[25] however, did not admit the influence of adrenals upon the alloxan initial hyperglycemia, for they observed the initial hyperglycemia even in adrenalectomized animals. In my experiment on the adrenalectomized rats, the initial hyperglycemia disappeared, the blood sugar fell immediately after the injection and the hypoglycemic convulsion appeared within several hours. These results show the important participation of adrenals to the alloxan initial hyperglycemia.

Then, to know what role the adrenal cortex or medulla plays in the alloxan initial hyperglycemia, the experiments of the demedullated rats were carried out. Firstly according to the results of histological studies of the adrenals on from 7th to 45th day after the time of demedullation, it revealed that not a small amounts of cortex are squeezed out with medulla at the time of operation, the regeneration of cortex takes place from the small amounts of zona glomerularis under the adrenal capsules, and its rate of regeneration is related to the number of days after the operation. The regeneration of adrenals is found still poor within 10 days after the time of operation but after that it reaches to the point that two or three layers of the adrenals can be distinguished with only few exceptions. And in some cases the adrenals are found hypertrophic in order to compensate the removed adrenal. The changes of the blood sugar after the alloxan injection in these rats depend on the existance of the medulla or the rate of regeneration of the cortex. Namely, in the cases with poorly regenerated adrenal cortex, the initial hyperglycemia disappears and the blood sugar always fall immediately after the alloxan injection and this change of blood sugar is independent upon the existance of the medulla. The rat with regnerated cortex shows the triphasic reaction of the blood sugar as normal rats do if their medulla remained, but in cases without medulla, the blood sugar falls during from 30 minutes to 2 hours after the injection of alloxan and then the triphasic reaction appears. From these experiments, it seems that the adrenal cortex provides an essential factor in the production of the initial hyperglycemia, and the adrenal medulla plays an important role for the initial hyperglycemia starting immediately after the alloxan injection.

Goldner and Gomori[5] presented the evidence in support of the hypothesis which takes the initial hyperglycemia as an expression of the effect of adrenalin. They showed that in rabbits in which adrenal medulla was destroyed by injection of formalin, the initial hyperglycemia failed to appear as seen in adrenalectomized rabbits. Hughes *et al.* published the same opinion. On the contrary, Kirshbaum *et al.*[6] described that the production of alloxan initial hyperglycemia is due to the existence

of the adrenal cortex, for they found that the initial hyperglycemia disappears in adrenalectomized rats, while the blood sugar curve after the alloxan injection in demedullated rats does not show any difference from the normal. Iversen[9] also reported that in rabbits with the adrenal medulla destroyed by the injection of formalin or by thermocautery, the blood sugar after the alloxan injection showed the triphasic reaction as seen in normal rabbits. However, as Kirshbaum *et al.* determined the blood sugar only at intervals of from 2 to 4 hours, it may be said that there is a peril to overlook the transitory changes of the blood sugar immediately after the alloxan injection. Author feels that it is very difficult to destroy selectively the adrenal medulla alone by the injection of formalin or by thermocautery as having been done in Iversen's report. At any rate, above mentioned results are not entirely in accordance with the result of present study.

As noted above, the pituitary and the adrenal gland play an important role in the production of the alloxan initial hyperglycemia, but how and what degree these organs are working in this hyperglycemia remained unknown. As results in this study are obtained by the removal of these organs, next problem to answer is whether or not the initial hyperglycemia in normal animals is an expression of the intensified activities of these organs. In order to investigate this question, the ascorbic acid concentration in the adrenals of the rats after the alloxan injection was studied. (Table III). The difference in the concentration of the adrenal ascorbic acid between normal rats and the rats killed 2 hours after the time of the injection of physiological saline solution is statistically insignificant. But the difference between above two groups and the rats killed 2 hours after the alloxan injection is extinct and statistically significant. It is a confirmed fact that ascorbic acid has a close relationship to the secretory activity of the adrenal cortex.[26] In general, increased adrenocortical activity is associated with the reduction in the concentration of adrenal ascorbic acid.[26-30] It is generally recognized that in the healthy rats the administration of adrenocorticotrophic hormone (ACTH)[17),27),28),31),32] or the application of stress[26),33-35] immediately causes the reduction of the adrenal ascorbic acid without fail. Thus reduction of ascorbic acid is considered a very reliable index of adrenocortical activity. According to the results of adrenal ascorbic acid concentration in 3 groups and our knowledge in these days, it seems that the injected alloxan, acting as so-called stress, brings about the reduction of the adrenal ascorbic acid, through the hyperfunction of pituitary adrenocortical system in normal rats.

From the results noted above, it seems that disappearance of the alloxan initial hyperglycemia is due to the lack of secretion of ACTH in

hypophysectomized rats, and due to the lack of secretion of adrenocortical hormone (ACH) in adrenalectomized rats. I would like to suggest that in normal rats, the hyperfunction of pituitary adrenocortical system provoked by the alloxan injection is at least an important factor to bring about the initial hyperglycemia.

What mechanism cuases the transitory fall in the blood sugar during the period of 30 minutes to 2 hours after the alloxan injection in demedullated rats? Previously Shipley and Beyer[36] confirmed the existence of an initial phase of hypoglycemia following intravenous administration of a diabetogenic dose of alloxan to normal dog, and surmised that this phase is caused by endognous insulin. Wrenshall *et al.*[14] observed the same phenomenon in some cases of the fasted dogs anesthetized by the choloralose or sodium amytalum, and suggested that this initial hypoglycemia may be caused by a stimulating action of alloxan on the islets of Langerhans. In normal rat, however, there has been no report pointing to phenomenon. Similarly, present study failed to confirm any evidence to support the observations reported by Shipley *et al.* and Wrenshall *et al.* Now, according to the knowledge in these days, immediately after stress, there is seen an elevation of the epinephrine concentration in blood[27),37-39], which brings about the discharge of ACTH from the pituitary[40-44] causing more secretion of ACH that increases protein catablism, the formation of new carbohydrate and thereby produces the elevation in blood sugar under certain circumstances.[45-49] These processes expire in very short time, while it is known that the lack of epinephrine brings considerably delayed reaction.[27),28] Of course, it can not be said that the lack of epinephrine would explain all the facts in present study on demedullated rats. However, it may be said at least the epinephrine has some connection with those phenomenon observed. Namely, the injected alloxan seems to constitute the so-called stress in normal animals and produce the discharge of epinephrine following the hyperactivity of pituitary adrenocortical system. The delay of these responses is brought by the lack of epinephrine secretion. In former case the initial hyperglycemia appears immediately after alloxan injection while in latter case initial hypoglycemia comes first before the initial hyperglycemia appears.

In cases with the hypertrophically regenerated cortex the initial hyperglycemia was superimposed by the permanent hyperglycemia without accompanying with the secondary hypoglycemic phase. Previously Gorden[50] observed that the decreased ascorbic acid in adrenals in demedullated rats returned to the normal value on 32th day after the time of operation, Brownell and Fartman[51] reported that about 30 days after the same operation the glyconeogenesis of adrenal cortex increased to three times as high as normal levels. So it seems reasonable to conclude

that in these rats with the hyperactive pituitary adrenocortical system, the rate of glyconeogenesis produced by the alloxan injection is larger than that of normal rats, and owing to this, the appearance of the permanent hyperglycemia which usually occurs about 24 hours after the alloxan injection in normal rats is accelerated and obscures hypoglycemic phase which is said to be observed about 4 hours after the alloxan injection in normal rats.

But farther study is necessary to answer the question, whether the alloxan initial hyperglycemia is produced only by the hyperactivity of pituitary adrenocortical system or not.

Summary

1. In hypophysectomized rats, the injection of alloxan does not cause the initial hyperglycemia and the blood sugar always falls immediately after the injection with far more marked degree than in the hypophysectomized non-alloxan-injected rats. The hypoglycemic phase is very severe and often fatal in spite of repeated administration of large amounts of glucose. But in incompletely hypophysectomized rats (1/6–1/7 of the anterior pituitary lobe remained), the typical triphasic reaction of the blood sugar as seen normal rats, is observed. So it is considered that the pituitary is playing an important role in the alloxan initial hyperglycemia.

2. The reactions obtained in adrenalectomized rats are just same with that in hypophysectomized rats. In the group of completely demedullated rats with non-regenerated cortex the curve of the blood sugar are similar to that of the adrenalectomized rats, while in the rats with regenerated cortex it is lowered for the period of 30 minutes to 2 hours starting immediately after the alloxan injection, and then the triphasic reaction follows. Especially in cases with hypertrophically regenerated cortex the initial hyperglycemia is superimposed by permanent hyperglycemia without having secondary hypoglycemic phase. These facts reveal that the adrenal cortex is an essential factor to produce the initial hyperglycemia and the role of the adrenal medulla is to start this initial hyperglycemia immediately after the injection of alloxan.

3. The alloxan produces the significant depletion of ascorbic acid in adrenals of normal rats. So it seems that the alloxan acts as so-called stress which brings about the hyperfunction of pituitary adrenocortical system in normal rats. The author feels that this factor must not be overlooked to understand the mechanism of the alloxan initial hyperglycemia.

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Studies on the Mechanism of Alloxan Initial Hyperglycemia

II. Role of the Liver in Connection with the Effect of Alloxan on the Blood Sugar

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In the previous report[1] it was stated that the alloxan initial hyperglycemia disappeared in hypophysectomized or adrenalectomized rats, and alloxan acted as so-called stress which produced the hyperactivity of pituitary adrenocortical system in normal rats. Namely, it was demonstrated that this system was so closely related to the initial hyperglycemia. If it proves to be the case, whether or not the rapid and high alloxan initial hyperglycemia is produced only by the hyperactivity of this system, is still unknown. The purpose of this paper is to discuss this problem.

At first, studies of the influence of ACTH on the blood sugar revealed that the over-secretion of ACH which is produced by the injection of ACTH, can not be the only cause of the hyperglycemia. Among other possible factors, the relationship of the liver to the effect of alloxan on the blood sugar was investigated, because the liver plays an important role in the carbohydrate metabolism. Secondly an inquiry was done as to whether the fall of blood sugar starting immediately after the injection of alloxan in hypophysectomized or adrenalectomized rats in my previous report is the specific effect of alloxan. These results, together with the results of the previous studies, will be discussed summarily in an effort to explain the mechanism of alloxan initial hyperglycemia.

Experimental

Materials and Methods

The first experiment was done on 21 rats, the second in 25 rats weighing from 170 to 220 g., the third on 22 rabbits weighing from 1.7 to 2.2 kg.

The first experiment:—Normal rats which were fasted for 12 hours and the adrenalectomized rats which were fasted for few hours after waiting one to three days after the time of operation were put in ice box (O°C)

for 30 minutes to apply cold-stress. The blood samples were taken immediately before and after the exposure to cold stress and every 30 minutes or 1 hour during next 10 hours. The ascorbic acid in adrenals was determined in 7 normal rats killed 2 hours after the exposure.

The second experiment:—After the fasting for 12 to 18 hours, 14 normal rats were given 1 or 2 mg. of ACTH (Amour) intraperitoneally. The blood samples were taken from the tail vein every 30 minutes or 1 hour during 12 hours. The concentration of ascorbic acid in adrenals was determined in rats killed 2 hours after the injection of ACTH.

The third experiment:—The 5 per cent solution of alloxan monohydrate was prepared and injected into the marginal vein of the ear in dose of 100 mg. per kg. of body weight. (This dose of alloxan is sufficient to produce diabetes in rabbits.) Experiments were studied in the following three groups.

(1) The seven rabbits were studied. After a vertical incision was made in the middle line of the abdomen, the hepatic artery and the portal vein were exposed and then the ligation was made near the hepatic hilus. Immediately after the ligation, the alloxan was injected, and 5 minutes later, the knot was removed and the incision was closed.

At the same time, a small portion of the tail of the pancreas was knotted immediately before the injection of alloxan. The ligation was also removed 5 minutes later. Histological study was made on this part of pancreas which had a temporary occulsion of blood stream and a part of the remaining pancreas which were fixed in Bouin's fluid and stained by Gomori's method. (Group A)

(2) The abdomen was opened in a same way. Five minutes after the injection of the same dose of alloxan, the hepatic artery and the portal vein were ligated temporarily for 5 minutes, and then the incision was closed. (Group B)

(3) After the abdomen was opened by similar procedure, the same dose of alloxan was injected into the ear vein and 5 minutes later the incision was closed (Group C).

The estimation of the blood sugar was made just before and every one hour after the injection of alloxan by Hagedorn-Jensen's method.

Results

The results of the first experiment are shown in Table I and II. Normal rats show the slight hyperglycemia immediately after the exposure to cold stress but the value returns quickly to the initial level and maintains the normal level during 10 hours. And the average value of ascorbic acid in adrenals in 7 cases killed 2 hours after the exposure is 241.6± 31.87 mg. per 100 g. of adrenal which is less than normal values. While

in adrenalectomized rats, the blood sugar falls immediately after the exposure and then shows a gradual elevation, which, however, does not return to the initial level even after 10 hours.

TABLE I

Average Value of Blood Sugar in Normal Rats after Injection of ACTH in doses of 1 or 2 mg., and in Normal and Adrenalectomized Rats after the Exposure to Cold Stress

(O°C, 30 minutes)

Group	No. of rats	Blood sugar value (mg/dl)												
		Bef.	0	½	1	1½	2	3	4	5	6	7	8	9	10hrs.
ACTH normal	14	98	—	94	99	97	98	99	96	95	97	96	95	96	96
Cold stress normal	6	98	125	107	97	98	95	99	95	97	95	95	95	94	94
" adrenex	8	89	59	56	58	61	64	68	70	71	77	75	78	78	82

TABLE II

The Adrenal Ascorbic Acid Concentration in Normal Rats and in Rats Sacrificed 2 Hours after Injection of Various Agents or Exposure to Cold Stress

Group	No. of rats	Adrenal ascorbic acid concentration (mg. per 100 g. of adrenals)
Normal	10	393.8±32.14
ACTH 1 mg per rat	6	316.5±20.61
" 2 mg per rat	5	256.6±31.46
Alloxan 180 mg per kg. of body weight	10	229.0±19.68
Physiological saline solution same amounts of alloxan solution	10	388.0±41.99
Cold stress 0° C, 30 min.	7	246.1±31.87

The results of the second experiments are shown in Table I and II. While the blood sugar in normal rats which receive 1 or 2 mg. of ACTH intraperitoneally does not show almost any change, the average values of the ascorbic acid in adrenals in two groups of rats, one of which is killed 2 hours after the injection of 1 mg. of ACTH and the other after the injection of 2 mg. of ACTH, are 316.5±20.61 mg. per 100 g. of adrenal and 256.6±31.46 mg. respectively. Namely, two groups show the depletion of ascorbic acid concentration in adrenals as compared with normal rats, the larger dose causing the more decrease in the concentration.

Table III and Fig. 1 show the results of the third experiment. The normal rabbits respond with the typical triphasic reaction of blood sugar

TABLE III

Average Value of Blood Sugar Changes in Rabbits after Intravenous Injection of Alloxan in Doses of 180 mg. per kg. of Body Weight under Various Conditions

Group	No. of rabitts	Blood sugar value (mg/dl)												
		0	1	2	3	4	5	6	7	8	9	10	24	48 hrs.
Normal	5	97	169	237	210	139	115	93	80	67	60	58	135	269
A	7	100	103	98	77	70	67	68	61	55	53	51	122	205
B	5	93	172	215	228	161	127	90	76	71	61	49	165	258
C	5	106	156	212	188	127	93	81	70	72	62	57	164	249

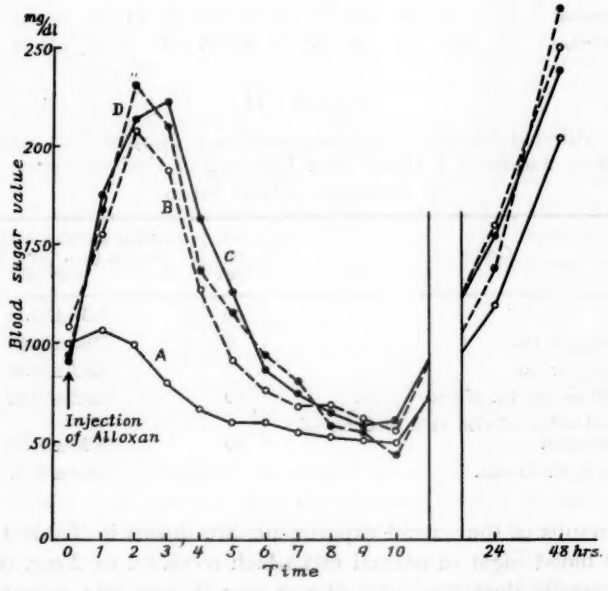

Fig. 1. Average value of blood sugar changes in rabbits after intravenous injection of alloxan under various conditions.

Curve A: rabbits whose liver were cut off from the blood stream for 5 minutes by temporary occulsion of hepatic artery and portal vein just after the injection of alloxan.

Curve B: rabbits whose hepatic artery and portal vein were ligated for 5 minutes, 5 minutes after the injection of alloxan.

Curve C: rabbits which were injected alloxan after opening of their abdomen.

Curve D: normal rabbits.

followed by the permanent hyperglycemia. The rabbits of group B and group C also respond with the same blood sugar changes as seen in normal rabbits. However, in one case in Group A, the blood sugar starts to fall immeditately after the injection of alloxan, and in 6 cases, the blood sugar rises slightly or does not show any change during 1 to 2 hours after the injection of alloxan and then gradually falls. It can be said that the initial hyperglycemia does not appear in this group and that the secondary hypoglycemic phase is severe and comes early as compared with other groups.

Pathological examination reveals that no significant histological changes are noted in the pancreas in which the blood stream is shunted temporarily by ligation, while in beta cells of the pancreas without this procedure, the pyknosis of the nuclei, disappearance of the granula and complete disappearance of beta cells are found as stated in previous reports.

Discussion

The concentration of ascorbic acid in adrenals in normal rats after being left in ice box (O°C) for 30 minutes shows a statistically significant reduction as compared to normal values. The difference of blood sugar curves after the exposure to the same cold stress between normal and adrenalectomized rats is very marked. Various reports have been made about the response of adrenals to stress. In general, the stress brings about a rapid increase of utilization of corbohydrate with the increased cellular activity,[2-4] and at the same time, produces the hyperfunction of the adrenal cortex accompanied by increased glyconeogenesis.[3-7] A slight hyperglycemia appears in certain circumstances.[8-10] Engel[4] states, "Since all types of stress are followed by evidences of increased protein catabolism and activation of adrenal cortex in normal but not in adrenalectomized or hypophysectomized animals, whereas stress regularly results in hypoglycemia and death in the latter." Previously I reported that in hypophysectomized or adrenalectomized rats the blood sugar always fell immediately after the injection of alloxan and the degree of the fall was more marked than the hypophysectomized or adrenalectomized rats without alloxan injection. Moreover, the alloxan produced the marked reduction of the asocorbic acid concentration in adrenals as seen in groups exposed to cold stress in present study. According to the Sayers's opinion[11),12)] that the degree of reduction of ascorbic acid concentration in adrenals is parallel to the strength of stress, it seems that the alloxan (180 mg. per kg. of body weight) and the cold (O°C, 30 minutes) are acting similarly as stress in rats. So it is considered that the immediate all of blood sugar after the injection of alloxan has not been necessarily

produced by the specific effect of alloxan, but by the general effect of stress.

Though the significant reduction of the concentration of ascorbic acid in adrenal in rats after the injection of 1 or 2 mg. of ACTH is understood there is an accelerated secretion of adrenocortical hormone (ACH), the blood sugar does not show any change. According to the recent progress, ACTH is diabetogenic, but the diabetic syndrome appears only after repeated injections of ACTH for a long time.[21-26] Sayers[13] reported that in 2 healthy persons who were given 50 mg. or 100 mg. of ACTH, the blood sugar did not show any change. Forsham *et al.*[14] confirmed same result in Addison's disease. From these results, it is likely that ACTH does not have a phramacologic action as does the epinephrine that produces the increased mobilization of glucose from liver and thereby the elevation of blood sugar. It was pointed out in the previous report that the alloxan initial hyperglycemia was closely related to the pituitary adrenocortical system, the hyperfunction of which was brought by the injection of alloxan. According to above observations, however, it seems that the alloxan initial hyperglycemia is not produced only by the hyperactivity of pituitary adrenocortical system and there must be some other unknown essential factors working in this mechanism. In general, the animal does not always respond to stress with hyperglycemia,[15),16] and even when hyperglycemia is found, its degree is slight as seen in the exposure to cold stress. So it may be suggested that the very rapid and high initial hyperglycemia is not only due to the general adaptation syndrome which is caused by the exposure to stress, but also due to the specific quality of alloxan which comes from its chemical structure and property.

To make clear this question, a role of liver in connection with the effect of alloxan on the blood sugar is investigated in rabbits.

In normal rabbits which are injected alloxan in doses of 100 mg. per kg. of body weight, the blood sugar shows the typical triphasic reaction followed by permanent diabetes. This dose of alloxan is the same amount which Banerjee[17] and Kendall *et al.*[18] used in their experiments.

It is known fact that the alloxan disappears very rapidly from the blood stream in animals. Leech and Bailey[19] reported that alloxan is resolved and deprived of diabetogenic action within 5 minutes after the time of injection. Goldner and Gomori[20] confirmed the same results within 6 minutes after the injection. The histological findings in the third experiment demonstrate that the diabetogenic action of alloxan disappears within 5 minutes after the time of injection as reported previously, even when the liver is cut off from the blood stream. So it seems that there is no close connection between the rapid disappearance of the

specific action of alloxan which destroys the beta cells of islets of Langerhans and the liver which is an important organ with antidotal action in body.

The blood sugar curve after the injection of alloxan is especially very noteworthy in Group A. Namely, in Group A in which the liver undergoes a temporary occulsion of hepatic artery and portal vein by ligation for 5 minutes after the injection of alloxan, the alloxan initial hyperglycemia disappears completely or is supressed markedly. In this observation there remains a doubt that this phenomenon may be connected with physiologically abnormal condition caused by operative procedure. However, this doubt could be wiped out by the experiment in group C where the blood sugar changes after the injection of alloxan was proved to be as same as normals. Furthermore, as the ligation of hepatic artery and portal vein means a complete occulsion of all blood stream to liver even though the duration is limited to only 5 minutes, possible explanation at this stage is that this obstruction of the blood stream in the liver might cause the depressed function of liver cells, or, that an entire mechanism of producing by some other elements which come out from the other organs except affected liver and work on the liver might also be disturbed by this operative procedure. But next experiment in group B revealed some facts against existence of such disturbances. It can be said therefore, the chief cuase of the disappearance of the alloxan initial hyperglycemia in group A must be pursued in the fact that the liver was protected from the aggression of alloxan by the temporary occulsion of the blood stream to liver.

If it proves to be the case, what do these results mean? Some investigators studied on the role of liver in connection with the alloxan initial hyperglycemia. Houssay *et al.*[27] demonstrated that the initial hyperglycemia was due to a direct effect of alloxan on the liver, for they found that this hyperglycemia disappeared in hepatectomized dogs and intensified when alloxan was directly injected into vena protae instead of vena saphena. Canzanelli *et al.*[28] opposed to the Hussay's opinion, because they found that the alloxan did not have any direct influence on the mobilization of liver glycogen in vitro. Bhattacharya[29] and Okamoto[30] observed that in animals which were injected alloxan or oxin after the livers were destroyed by CCl_4 or chloroform, the initial hyperglycemia was found as in normal animals. In present study, the alloxan initial hyperglycemia does not appear in Group A in the third experiment in which the direct action of alloxan on the liver can be completely excluded, for alloxan has already disappeared from the blood stream at the time when the knoting are cut off 5 minutes after the time of injection. Moreover, all rabbits of group A became permanent diabetes. According

to these observations, it seems that the alloxan has an action which produces the mobilization of liver glycogen.

The alloxan destroys specifically, selectively and progressively the beta cell of islets of Langerhans immediately after the injection. So Duff,[21] Iversen[32] and Okamoto[30] mentioned that the blood sugar changes after the injection of alloxan were due to this character of alloxan and the initial hyperglycemia corresponded to the relative dominance of the antiinsulinic hormones of anterior pituitary lobe and adrenal cortex caused primarily by the lack of insulin secretion from the islets of Langerhans destroyed by the alloxan. But Banerjee,[32] Goldner,[33] Bailey[31] and Wrenshall[11] opposed this hypothesis, for they found the initial hyperglycemia even in pancreatectomized dogs or in rabbits with temporary occulsion of arteries to pancreas. Moreover, the injection of ACTH,[13][14] growth hormone[36–39] or ACH which has antiinsulinic effect, does not produce any hyperglycemia as seen in the first experiment in the present study. So it is not likely that the alloxan initial hyperglycemia is due to the lack of insulin secretion.

The mechanism of alloxan initial hyperglycemia, a very interesting problem in studies on alloxan diabetes, is still left unresolved though many reports have already been made. An inquiry into this problem has been attempted in this paper by discussing the results obtained. Its conclusion is as follow.

Firstly, the adrenal cortex, the adrenal medulla, the pituitary and the liver are respectively indispensable important factors in the production of the alloxan initial hyperglycemia. Secondly, there is a close interelationship between them. Removal of any one of these organs, would mean instant disappearance of the alloxan initial hyperglycemia. Thirdly, the mode of their actions is understood as follows. The pituitary and adrenal gland act as one system, reaction of which is the general response of the animal body to stress. The liver responses specifically to alloxan in the mobilization of glycogen. So the mechanism of alloxan initial hyperglycemia can be depicted as a schema as shown in Fig. 2. The initial hyperglycemia is produced by the hyperactivity of pituitary adrenocortical system following the administration of the alloxan, which, at the same time, works on the liver, and mobilizes the sugar from liver glycogen. Moreover, under the existence of adrenal medulla, the initial hyperglycemia starts immediately after the injection of alloxan, and this is not observed when the adrenal medulla is removed. So it is considered that the role of the adrenal medulla is to start the initial hyperglycemia immediately after the injection of alloxan. Though it may be said that some other factors are working in this mechanism, because of various toxic actions of the alloxan,[40] especially the action on beta cells of islets

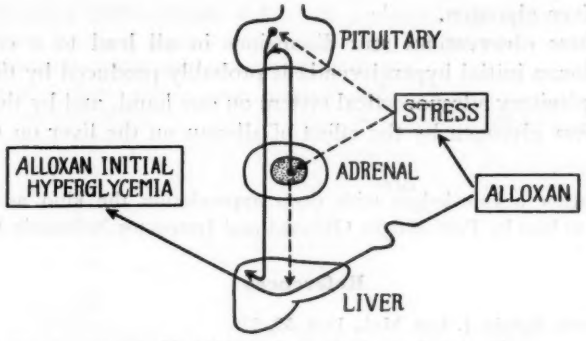

Fig. 2. Diagram of mechanism of alloxan initial hyperglycemia.

of Langerhans and the action on the carbohydrate intermediate metabolism, etc. It is believed that the chief factors in the mechanism of producing the alloxan initial hyperglycemia are found in the ground principle as shown in Fig. 2.

Summary

1. The significant reduction of the concentration of ascorbic acid in adrenals in normal rats 2 hours after the exposure to cold stress (0°C, 30 minutes) was observed. Normal rats showed the slight hyperglycemia just after the exposure, but the blood sugar returned soon to initial level and held the normal level during 10 hours. In adrenalectomized rats, the blood sugar fell just after the exposure, then turned to upward during 2 hours but did not return to the initial level even after 10 hours.

2. Although the ascorbic acid concentration in adrenals in rats showed a significant reduction when 1 or 2 mg. of ACTH was given, the fasting blood sugar did not show any changes. So the hyperactivity of pituitary adrenocortical system which is produced by the alloxan can not be the single cause of the initial hyperglycemia.

3. The diabetogenic action of alloxan disappears within 5 minutes after the time of injection, without any possible involvement of the liver.

4. In normal rabbits which were injected alloxan in dose of 100 mg. per kg. of body weight, the response is the typical triphasic reaction of the blood sugar followed by permanent diabetes. However, when the liver is stunted from the blood stream for 5 minutes after the injection of alloxan by temporary ligation of hepatic artery and portal vein, the alloxan initial hyperglycemia is completely gone or at least markedly repressed. As the influence of operative procedure can entirely be excluded, it is suggested that alloxan has an action to the liver and produces the mobili-

zation of liver glycogen.

5. These observations and discussions in all lead to a conclusion that the alloxan initial hyperglycemia is probably produced by the hyperactivity of pituitary adrenocortical system on one hand, and by the mobilization of liver glycogen by the effect of alloxan on the liver on the other hand.

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Utilization of Chitinase in Treatment of Dermatomycosis

I. Influence of Bacilli chitinovori on the Culture of Dermatophytes

By

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The reason why dermatomycosis is apt to repeated relapse and thus is hardly conquered seems to lie in the fact that the cell membranes of the fungi are composed of the extremely stable compound chitin. Thus, to break through the deadlock in the progress of therapy of dermatomycosis, we are driven to the belief that we must either apply some medicine that would permeate through such cell membranes or would decompose them to reach the inner cell bodies of the fungi and kill them. From such a point of view, and taking the second alternative, I undertook to try chitinase for the decomposition of the chitin membranes. For the purpose of preparing chitinase I chose *bacilli chitinovori* as my material and asked Yamamoto[1] to collect and study them bacteriologically. In this report, the author will describe the results of the experiments on the influence of chitinase on the culture of dermatophytes as follows.

Experimental

Material and Method

I chose several types of *bacilli chitinovori* at random from the 57 types comprising 211 strains collected by Yamamoto for my materials. As for the dermatophytes, the following 7 types of them were placed at my service by Dr. Yoshisada Takahashi, who was then Assistant Professor of Tokyo University and now Professor of Juntendo Medical School: namely, *trichophyton ferrugineum*, *trichophyton violaceum*, *trichophyton gypseum*, *trichophyton asteroides*, *trichophyton interdigitale*, *epidermophyton inguinale* and *sporotrichum Beurmanni*. I herewith express my heartiest thanks to him for his donation which made my experiments described hereunder possible.

As solid culture media. I used slanting and plane surfaces of simple agar or agar in 4% glucose solution, and 4% glucose bouillon and Benton's culture medium[2] freed of chitin as liquid culture media.

The dermatophyte fungi and the *bacilli chitinovori* were transplanted for cutlture on the solid media either simultaneously or in succession, while in hanging drop cultures, both the microbes were always transplanted simultaneously. In all cases, the cultures were incubated at room temperature of 20–25°C or in an incubator at 37°C during the period of observation.

Results

1) With solid culture media:

a) Glucose agar; When the fungi and the *bacilli chitinovori* are transplanted thereon at the same time, the former thrive admirably while the latter grow more slowly in comparison. However, when slant surfaces of the media are used, the chitin decomposing bacilli begin to grow in approximately 3 weeks after the transplantation at the part where the condensed water collects and at this area the two types of the microbes come in contact, the carpet of the growing fungi is pushed up off the surface of the medium. When the transplantation of the fungi is put off till the previously transplanted *bacilli chitinovori* have grown in a dense mass, a similar peeling of the film of the fungi was observable. When the fungi are grown beforehand to cover the surface of the medium, the subsequently introduced bacilli fail to mature into colonies.

b) Simple agar; When both the fungi and the bacilli are transplanted simultaneously, the latter grow rapidly and take up all the surface of the culture medium, so that no growth of the fungi is possible. When the fungi are first grown and the bacilli transplanted on a medium half covered with the growth of the fungi, the film of fungi shows the appearance of being rolled up at the edge where it comes in contact with the colony of the bacilli. The fungi turn brownish at the top and this anomaly continues for some length of time in existence.

Upon microscopic examination of such rolled-up fungi, with or without staining, such as by Gram's method, it is observed that the mycelia are shreded and the free edges of the cell membranes are minced. At the early stage, such impaired fungi react negatively against Gram's staining, but with the progress of the degreneration, the part where presumably the protoplasm is located turns positive in Gram's staining, then it crumbles down into amorphous masses, finally losing all protoplasm, only the cell membrances remaining to appear unstained in the figure of 'ghost.'

In no case, we observed any difference in such results due to change in the temperature during the examination or the variety of the strains of either the fungi or the bacilli used in the test.

2) On hanging drop cultures:

a) When glucose bouillon was used as medium, the fungi prospered rapidly and lustily, while the chitinoverous bacilli lagged behind in growth, but no other mutual effect was observed between the two strains.

b) When simple bouillon was used, the chitinovorous bacilli thrived better. They densely surrounded the mycelia and the spores of the fungi and we observed breaches in the cell membranes, the free edges of which were torn into shreds. The protoplasm was first negative to Gram's staining, but turned positive later on as amorphous masses which finally disappeared, leaving only the cell membranes.

c) The use of the liquid constituent of Benton's medium freed of its chitin fragments prevents the growth of the fungi, and the results were similar to that described in b) above. In particular, such results can be clearly observed when large-sized fusiform spres are used for the culture of the fungi.

In no case any difference in the results was observed due to change in temperature and variation of the strains of the fungias well as of the bacilli.

Summary and Discussion

The author undertook to try a way to overcome the deadlock in the treatment of dermatomycosis by decomposing chitin, the chief component of the cell membrane of fungi, and for this purpose, began with a test in a mixed culture of dermatophytes and *bacilli chitinovori*. The results showed that it was very difficult to experiment at once on two microbes with different conditions for growth under the same experimental conditions. That is, when one of the tested microbes is grown under its optimal condition, it will take up and cover the whole surface of the medium before any trace of growth of the other, less favored microbe becomes observable. Only under the following conditions could any effect of contact between the growth of the two microbes be observed, namely, when *bacilli chitinovori* were introduced on simple agar medium where the previously planted dermatophytes were in growth to some extent, and when hanging drop culture was effected by simultaneously planting of the fungi and the *bacilli chitinovori* on simple bouillon medium. In the latter case, the picture of the chitinovoruos bacilli attacking the chitin-armored fungi was observable with special clarity. The attack consisted in a tight seizure of the fungi by the bacilli, which caused a rupture in the cell membranes of the former, laying bare their protoplasm, which turned from Gram negative to positive and finally disappeared entirely, leaving an army of 'ghosts' of fungi. Such a scene may be traced to

best advantage when large-sized fuseaux of the fungi are cultured on the liquid portion of Benton's medium freed of chitin, together with the bacilli. Such phenomena undoubtedly show that dermatophytic fungi are subject to invasion by chitinovorous bacilli, but seeing that the above tests where the defeat of the fungi at the hand of the bacilli was enacted were conducted under conditions unfavorable for the former, I fear it is premature to deduce such optimistic views of successful victory over the tenacious dermatophytes from the results of such tests alone.

I must mention with gratitude that the above study owes much to the Scientific Research Fund allocated by the Education Ministry for its completion.

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II. Influence of Crude Chitinase Fluid on the Culture of Eungi

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In the previous report,[1] the author has described the results of his experiments on mixed cultures of dermatophytic fungi and chitinovorous bacilli, mentioning the observed decomposition of the chitin cell membranes of the fungi and their reduction to the state of 'ghost.' However, as these experiments were conducted with two kinds of microbes entirely different in their conditions of successful growth as objects, their results may be open to criticism. Accordingly, I proceeded to try the effect of the crude chitinase fluid obtained from *bacilli chitinovori* on culture of dermatophytic fungi.

Experimental

Materials

1) Preparation of crude chitinase fluid: Benton's culture medium[2] was put in a 1,000 cc. culture flask, so that the maximum surface of the medium was obtained (the volume of the medium in this case amounted to about 700 cc.) and Yamamoto strain No. 190[3] of *bacillus chitinovorus* was cultivated on it, under aeration with a water-jet aspirator and at room temperature of 20–25°C. After the lapse of about 3 weeks, when the chitin in the culture medium had been completely dissolved, the content of the flask was ground in a mortar, filtered through Chamberland L_1 and the filtrate used as crude chitinase fluid, at the same time its asepsis was established by test culture.

2) The strains of dermatophytes used in the study were the undermentioned seven, kindly placed at the disposal of the author by Prof. Dr. Y. Takahashi of Juntendo Medical School. Deep appreciation is due to him for his donation. The strains were, namely (1) *trichophyton ferrugineum*, (2) *trichophyton violaceum*, (3) *trichophyton gypseum*, (4) *trichophyton*

asteroides, (5) *trichophyton interdigitale*, (6) epidenmpohyton inguinale (7) candida albicans.

Method

1) By formation of inhibition area of growth: At the center of a glucose agar plate entirely smeared over with an emulsion prepared from fungi suspended in physiological saline about tenfold in volume, and ground in a mortar are placed 4 disks of filter-paper 1 cm. in diameter and saturated with curde chitinase fluid. This plate is placed in an incubator kept at 37°C for culture.

2) By partial immersion: Fungi were cultivated on glucose agar plate held in a slanting position according to the idea of Szybalski[4] and after the growth spread over the entire surface of the medium, crude chitinase fluid was added so that about the half of the growth was immersed in the fluid. This was kept in an incubator at the temperature of 37°C for observation.

As a control in the above tests, a similar culture to which Benton's medium freed of chitin was added instead of crude chitinase fluid, was used.

Results

1) The inhibition area of growth formed by the method of 1) above was very limited, showing hardly any difference from the control.

2) In the test by method 2) above, the fungi immersed in the crude chitinase fluid were seen dissolved off the medium, leaving a bare surface at the end of third day of experiment. All the tested strains of the fungi showed the same dissolution.

A microscopic examination of the impaired fungi stained by Gram's method revealed that their mycelia and cell membranes were tattered, their protoplasm exposed and jumbled up in Gram-poistive amorphous lumps or otherwise reduced down to 'ghosts' of mere cell membranes devoid of any stainable substance. However, when the medium is left for a longer time in the incubator, the unaffected fungi continue to grow, so that in about a week, they begin to invade the part immersed in the crude chitinase fluid.

Summary and Discussion

In the experiments for observing the influence of crude chitinase fluid on the culture of dermatophytes, the author failed in producing an area in which the growth of the dermatophytic fungi was inhibited by means of filter-paper saturated with the fluid, but when a culture of the

fungi was immersed in the fluid, the fungi were nearly completely destroyed in three days, leaving 'ghosts.' In other words, chitinase in crude solution may be said to have *in vitro* the power of decomposing the cell membrane of the dermatophytic fungi and killing them. The fact that some seven days later the remaining culture develops into the part previously laid bare by the fluid seems to show that chitinase as the effective factor was gone by that time, either by being used up, by decomposition under the temperature of 37°C over a long time, or by loss of its activity through the influence of the decomposition products of the killed fungi or the metabolism excretion of the surviving fungi. Whatever the cause of such a subsequent loss of efficacy, I am led to believe that a crude chitinase fluid prepared from chitinovorous bacilli has the power to decompose the cell membranes of dermatophytic fungi and kill them, at least *in vitro*.

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